Measuring honey bee feeding rhythms with the Beebox, a platform for nectar foraging insects

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Abstract:	In honey bees, most studies of circadian rhythms involve a locomotion test performed in a small tube, a tunnel, or at the hive entrance. However, despite feeding playing an important role in honey bee health or fitness, no demonstration of circadian rhythm on feeding has been performed until recently. Here, we present the BeeBox, a new laboratory platform for bees based on the concept of the Skinner box, which dispenses discrete controlled amounts of food (sucrose syrup) following entrance into an artificial flower. We compared caged groups of bees in 12h-12h light/dark cycles, constant darkness and constant light and measured average hourly syrup consumption per living bee. Food intake was higher in constant light and lower in constant darkness; mortality increased in constant light. We observed rhythmic consumption with a period longer than 24h; this is maintained in darkness without environmental cues, but is damped in the constant light condition. The BeeBox offers many new research perspectives and numerous potential applications in the study of nectar foraging animals.
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Amiens, october 9th

Cover letter

Dear Pr Lutz,

We are pleased to submit to Physiology & Behavior the paper entitled “Measuring honey bee circadian rhythms with the Beebox, an operant conditioning platform for nectar foraging insects”. Our paper uses an original method to measure insect feeding rhythms. Our protocol includes the delivery of small controlled amounts of syrup to bees with miniature peristaltic pumps. The pulsation of the pumps has been suppressed with a patented compensation algorithm. Our paper includes data in several light conditions to measure feeding rhythms. Our study is about behavioral neurosciences and concerns the modulation of behavior by environmental factors. That’s why we think our paper falls into the scope of P&B and we hope it will be considered as a potential paper to be published in the Journal.

Yours sincerely.

Michel Sokolowski

Measuring honey bee feeding rhythms with the Beebox, a platform for nectar foraging insects

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Declarations of interest: none

HIGHLIGHTS

- The BeeBox automatically records feeding time and occurrence in insects.
- This reveals circadian rhythm in feeding in bees.
- This rhythm is lost in animals kept in constant light.
- Constant light also induces higher mortality.

ABSTRACT

In honey bees, most studies of circadian rhythms involve a locomotion test performed in a small tube, a tunnel, or at the hive entrance. However, despite feeding playing an important role in honey bee health or fitness, no demonstration of circadian rhythm on feeding has been performed until recently. Here, we present the BeeBox, a new laboratory platform for bees based on the concept of the Skinner box, which dispenses discrete controlled amounts of food (sucrose syrup) following entrance into an artificial flower. We compared caged groups of bees in 12h-12h light/dark cycles, constant darkness and constant light and measured average hourly syrup consumption per living bee. Food intake was higher in constant light and lower in constant darkness; mortality increased in constant light. We observed rhythmic consumption with a period longer than 24h; this is maintained in darkness without environmental cues, but is damped in the constant light condition. The BeeBox offers many new research perspectives and numerous potential applications in the study of nectar foraging animals.

Key-words: honey bees, Skinner box, feeding patterns, circadian rhythms, mortality assessment, operant conditioning

1. INTRODUCTION

As many other species, honeybees are diurnal animals whose behavior and physiology are strongly affected by the cyclic dimension of their physical and biological environment. In many latitudes, the cyclic alternation of day and night has a strong impact on flower resources gathered by bees (mainly nectar and pollen), and flowers activity shows some kind of circadian rhythmicity [1]. For numerous flowers, nectar production and nectar sugar content depend on the time of the day [2–8] with a peak observed in the morning for some flowers [9,10] and in the afternoon for others [11,12]. Moreover, some flowers also show opening and closing cycles [13] that limit the availability of nectar and pollen. When evolution takes place in such an environment involving plant-pollinator interactions and co-adaptation, temporal match between honeybees and flower traits can be expected [14–16]. Consequently, it is not surprising observing bees visiting flowers when nectar is at its highest level [12,17,18]. Two kinds of behavioral phenomena can be potentially involved in this kind of temporal match: spontaneous circadian rhythms and learning [19–21].

Circadian rhythms refer to an endogenous oscillation in a biological variable that last approximately 24 h [19,22,23]. Such a rhythm is not learned, but entrained by some environmental signal, also called "zeitgeber" [24], that can be, for example, light, temperature, or food availability [19,25]. Entrainment refers to an active mechanism that synchronizes the biological variable to the cyclic occurrence of the "zeitgeber" [26]. It is often assumed that such rhythms may procure a selective advantage with the anticipation of some predictable and cyclic environmental important events [15,16] such as availability of flower resources for honeybees.

However, circadian rhythms are not the only mechanisms to adjust to time-dependent events. Learning allows anticipating periodic events, most often food [27]. Traditionally, temporal regulation has been studied by psychologists with pavlovian [28] or operant conditioning timing protocols [29], for example with single food items being presented at regular time intervals independently, or as a consequence, of some arbitrary response. Interval timing or temporal conditioning occurs if the animal anticipates the occurrence of the food event with no other cue than the time since the last food occurrence [30,31]. As demonstrated in vertebrates, circadian rhythms and temporal conditioning may involve a variety of physiological mechanisms [19,32,33] and may interact together [34]. Classic temporal conditioning involves seconds to minute intervals with time intervals initiated at any point of the day-night cycle [30] when circadian rhythm studies concern 24 h duration intervals. However, it has also been suggested that temporal regulation may happen over longer intervals in food-anticipatory activities [30,35]. In these studies, the protocol combines a circadian and a timing dimension: food is available only at a fixed point of the day-night cycle [30,36] and then removed. Such protocols differ from pure interval timing studies because food is not presented after a given

delay but is only available ~~during a~~ for 2-4 hours ~~period of time and~~ [37]. The task may be a little bit more complex if the protocol includes also a spatial dimension, in a so-called time-place learning protocol where the location of a food item or a meal depends on the time of the day [38,39], with one or more, locations available [40,41].

When food is presented periodically with a period of 24h, learning and circadian rhythms are two potential candidates to explain periodic responding. In order to be a relevant adjustment to environmental conditions, a circadian rhythm must be entrained by food occurrence, which then act as a "zeitgeber"[42]. If animals develop temporally conditioned or food-anticipatory responses, then food may become an anticipated unconditioned stimulus [37,38]. Both mechanisms could act alone, or combine their effects. [39].

In bees, most studies on circadian rhythms involved a locomotion test. Such test is most often performed with a bee encaged in a small tube or a tunnel equipped with infrared sensors [43–47], performed with video tracking [48], or at the colony entrance [49–51]. Yet, locomotion is only an indirect measure of food gathering; rather, honeybee health and colony fitness are directly related to nectar consumption [52,53]. Unfortunately, while circadian rhythms in locomotion have been studied, very few tests have studied feeding rhythms [54]. Feeding opportunity has been sometimes manipulated in food-anticipatory or time-place learning protocols [18,45,49,55,56] but food consumption was not measured, and studying foraging rhythms with bees is a quite different problem than studying individual food consumption because, nectar gathering during foraging activity does not reflect metabolic activity.

Yet, such links between feeding and rhythms seems to exist: for instance, female (but not male) flesh flies *Sarcophaga crassipalpis* exhibit an extended scotophase activity according to their nutrition (and their age), suggesting food intake can modulate circadian rhythm [57]. Similarly, food deprivation can alter sleeping patterns [58]. Such effects can be explained by an impact of food intakes on the gene expression balance that finely regulates circadian rhythms [59–61], such as *c~~C~~lock*, *period* and *timeless*. Indeed, in the sandfly *Lutzomyia longipalpis*, a blood meal can downregulate period and timeless gene expression levels [59]. Moreover, food intake during foraging can lead to pesticide contamination (especially in pollinators like bees), which is known to affect rhythms too [62,63].

Overall, these results point to the need for more studies on the rhythm of food intake in insects, or on the role of food in rhythm entrainment, especially in bees. The lack of feeding tests in honey bee rhythm research could reflect the lack of a proper technology to measure consumption in long periods. A protocol commonly used in feeding experiments involves small cages with nearly-unlimited and easily accessible food, most often syrup (concentrated sucrose solution) stored in a feeder [64–66]. Mortality and food consumption are assessed punctually, most often daily, by

respectively manual counting of dead bees and weighting the feeder [67,68] or measuring the level of food inside a tube [69]. However, such manual protocols do not allow fine studying of circadian rhythms because there is the need to measure consumption repeatedly (i.e. hourly) during several consecutive days. Automated feeding-measurement protocols have become popular in others species and several approaches have been developed to assess circadian feeding rhythms and record consumption repeatedly during the long term [70]. One of them involves a Skinner box, where food is delivered in small quantity provided animals release a specific response [71–73]. With such a device, the animal has to emit repeatedly the response to get its daily food ration [74]. Unfortunately, while already used for short duration free flying experiments with bees [75], or lab experiments with flies [76], previously used tools are inadequate in laboratory setting.

To fill this gap, we recently developed a new concept of laboratory Skinner box [54,77] able to deliver small controlled amounts of syrup to bees visiting an artificial flower. The box allows precise measures of food intake time and occurrence during several days. This paper follows three main objectives. First, we present our new automatized conditioning chamber, the "BeeBox". Second, using this new tool and a "true" circadian protocol with continuous access to food, we explore feeding rhythms in honeybees and whether these rhythms can be modulated by light conditions. Finally, we discuss the potential applications of our platform to deepen the study of circadian rhythms and to explore other fields related to ecological, behavioral, physiological and ecotoxicological research in nectar foraging animals.

2. MATERIAL AND METHOD

2.1 Animals

Experiment took place between April and June 2016 and later between end of September-beginning of October at the Université de Picardie – Jules Verne, Amiens (France). Honey bees (Buckfast) were caught on one frame of a single 10-frames Dadant hive located in the apiary close to the laboratory early in the afternoon. The frame was extracted from the hive and then kept vertical on a table with the help of a wooden support. Bees were then collectively captured with 30ml sample plastic containers and quickly introduced in the BeeBox without anaesthesia with the help of the sliding cover (see apparatus section). Each BeeBox was then randomly assigned to one of the three treatments (see below). This procedure was preferred to catching leaving or returning foragers for two reasons. First, foragers are known to be older bees. To illustrate the interest of the BeeBox for long-term measurements, our experiment was planned for at least 10 days; thus, we wanted to limit natural mortality due to age, leading us to avoid foragers. Second, we had to fill chambers with several hundreds of bees and had only a short time to perform this task. The collective capture of bees with

several 30 ml plastic containers took only few minutes and it would have taken much more time to catch foragers one by one at the hive entrance. This protocol does not control the exact age of the bees, but increases the probability to work with young bees; it also minimizes handling and stress, and avoids anaesthesia. Furthermore, bees are randomly assigned to each treatment. We did not capture drones and bees with pollen balls because drones have a larger size that do not fit to the size of the flower diameter and pollen balls could be stored inside the response hole and block the dispensing needle (see *Figure 1. A*); moreover, pollen balls are carried by forager bees. Studying foragers would definitely be important, as it is known forager and non-foragers bees have differing circadian rhythms [78].

2.2 Apparatus

The 12 identical BeeBox (overall dimensions: 20cm x 12cm x 25cm, L x l x h) were composed of three parts *(Figure 1 and Figure 2).*

The bottom part included the printed circuit board, a peristaltic pump (6 rollers Kamoer KCS stepper motor peristaltic pump), a small OLED display (Adafruit Monochrome 0.96" 128x64 OLED graphic display, https://www.adafruit.com/product/326), a three push button keyboard, the on/off button, and a 40mm x 40mm fan.

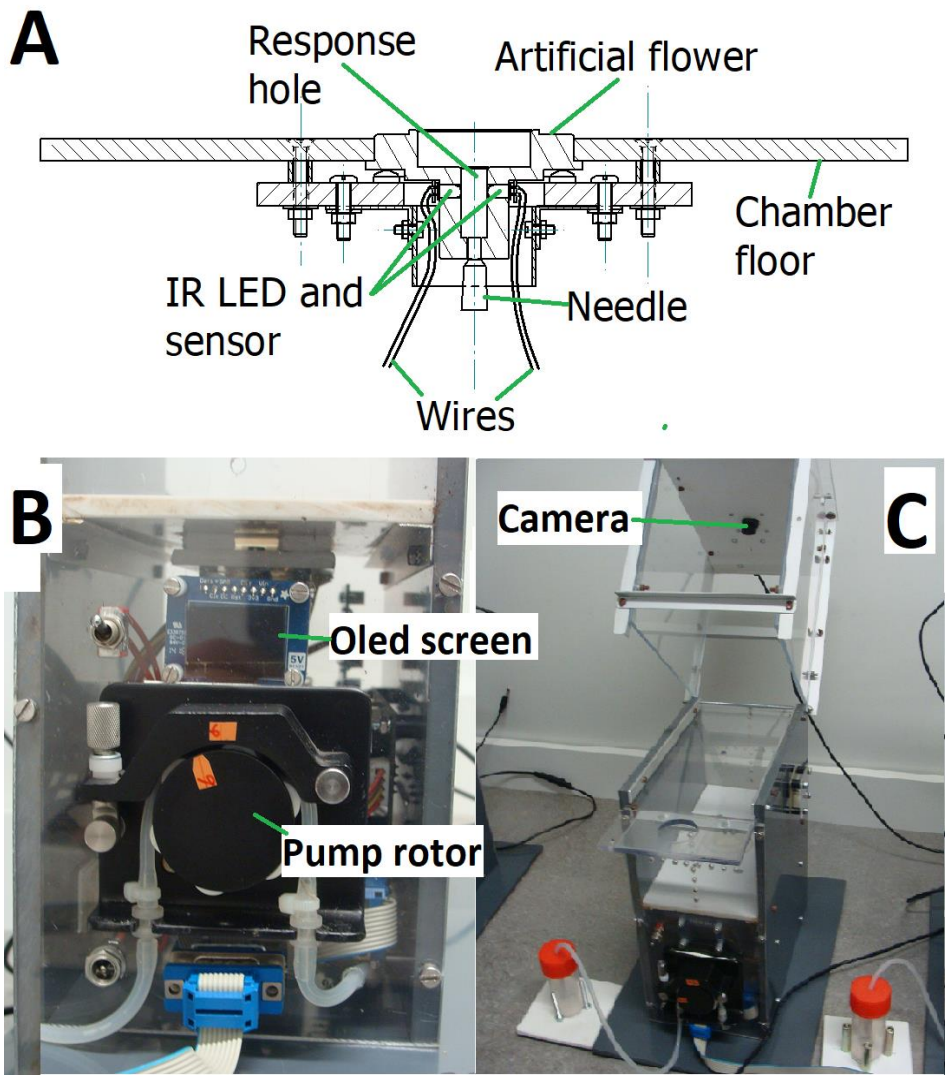

Figure 1. A. Drawings of a sectional view of the artificial flower fixed on the floor of the bee chamber. The IR LED sensors are connected to the pump controller. If needed, it is possible to add a 16 RGB LED ring under the flower to color it from below. The silicone tubing connected to the needle is not shown. **B.** Front view of the bottom part of the conditioning box showing the pump and the silicone tubing. Orange labels are used to set the reference position. **C**. BeeBox with the top camera part lifted. After being captured in tubes in the hive, bees are set in this central part without anesthesia by fitting the tube in a circular hole in the top of the box, and then sliding it over the box to release them. The syrup (sucrose solution used to feed the bees) is stored in the red cap 30ml plastic bottle.

The center part is the cage for the bees (20cm * 10cm * 9cm height). It had 32 3mm holes for ventilation and a transparent sliding cover. This cover allows to introduce awake bees from a standard 30ml sample container without any risk of escape. The front and back face were in transparent polycarbonate for visual observation, and the side faces in opaque PVC. The floor of the bee cage was removable for cleaning. The main part of the conditioning chamber was a 46 mm diameter cylindrical polyamide artificial flower machined with a lathe and screwed in the centre of the floor. In the centre of the flower, we drilled a 6mm diameter/17 mm long hole (i.e. approximate size of a bee) with a standard 25G Luer needle (needle cut flush to the cone) glued in the bottom for syrup dispensing. The surface of the flower contained a small 2mm high rim serving as an obstacle to prevent any dead bee from falling inside the flower opening.

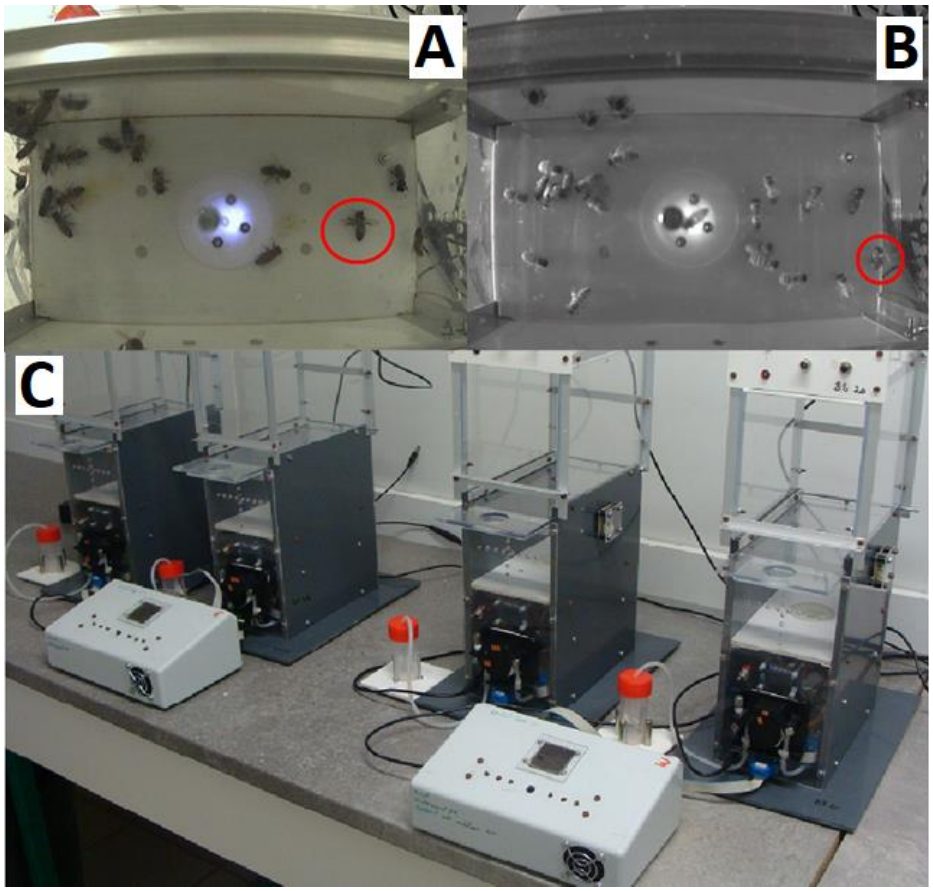

Figure 2. A. Sample picture for mortality assessment during the light-ON condition**. B.** Sample picture for mortality assessment during the dark condition. In each case, a dead bee (circled with red) can be identified. The bright spot visible in the center of the flower (panel A and panel B) corresponds to light from the IR LED. **C.** Picture of the experimental setup for each of the three rooms (four BeeBoxes in each). Each control interface drives two separate conditioning chambers.

The top part of the BeeBox is a swivel cover (20cm * 12cm * 21cm) equipped with a sensor camera (PTC08 serial JPEG camera with NTSC video) for mortality measurement. The serial camera was driven by an Arduino Uno board improved with a real time clock and a SD card reader (Adafruit Data Logging shield for Arduino) and programmed to take a picture each hour and to store it on the SD card. The devices were powered with a set of six standard 12V DC power modules that could power each two different conditioning chambers.

To get food, a honey bee had to enter in a vertical hole in the centre of the artificial flower; this entrance was detected with an IR Sensor (*Figure 1A*), which in turn triggered the release of food in a smooth and consistent way. These features correspond to the hallmarks of a Skinner box [75,79]: a well-defined behaviour (i.e., entrance in the tube) and a resource contingent to the production of this behaviour (food syrup, which is the reinforcement in the terminology of Skinner box protocol). It is also possible to add stimuli signalling the availability of the resource, such as light: a 16 RGB LED ring can be placed under the flower so that it is also possible to colour the flower from below and diffuse light into the polyamide disc. This allows to condition the food release to the presence of the appropriate visual background, but this was not used in the experiments reported here. Similarly, food could have been offered only during specific time [12,17,18], although this possibility was not used either in this work. The number of entrances required to release the food, as well as the dispensed amount of syrup, can also be controlled. This allows to control the effort required to obtain food; here, a single entrance was required. The BeeBox permits to continuously monitor food intake and mortality and controls the effort required to obtain food (i.e. the number of entrance) in a standardized way with reinforcement schedules, thus avoiding the need of experimenter's intervention. The approximate cost are 250€ for the conditioning chamber, 150€ for the top camera part and 250€ for the control interface, which gives a total of 650 €.

2.3 Syrup dispensing

A major technical problem with delivering small amount of liquid food with a peristaltic pump is the pulsation that is characteristic to this kind of pump because they use several rollers pressing a silicone tubing. Each time a roller takes off from the tube, reverse pumping occurs, and the pump dispenses nothing. Depending on the pump, the "no distribution" phase of the pump can vary until 30% to 45% of the total rotary cycle. Such problem prevents using this kind of pump to deliver with precision small discrete volumes of liquids (less than 1 µl). To solve it, we set a compensatory algorithm in the BeeBox controller working with stepper motor pumps. To measure and control the dispensed volume, we count the impulsions that are sent to the motor without counting impulsions when reverse pumping occurs [80]. Thus, initially the experiment pumps (Figure 1B) were manually

set to a reference angular position. The compensation amplitude was then measured empirically and depends on the pump and the tube properties. We used six rollers miniature pumps driven by a 200 steps motor with 0.8mm internal diameter silicone tubing, and a P8X32A microcontroller to drive the pump, monitor the angular position and compensate the "no distribution" phase. With one stepper motor step, the pumps dispense about 0.25µl [81,82]. However, larger volumes can be obtained with the successive activation of the stepper motor.

Our compensation algorithm paves the way for more applications using inexpensive peristaltic pumps to dispense small volumes of liquids and our device could be adapted to other species of nectar foraging animals like bumblebees [83], butterflies [84], moths [77,85], ants [86], or hummingbirds [87,88]. Moreover, even if the algorithm has been designed to dispense discrete amounts of syrup, the repeated activation of the stepper motor activated pump could also control low syrup flow rates [89].

To increase sample size in our experiment, we used cohorts of bees rather than single individuals. Testing individual bees would have generated a very low syrup flow with a risk of clogging the dispensing nozzle with dried syrup. Testing individual bees would need additional technical options to prevent this.

2.4 Mortality and food intake measurement

As there are several bees in each box, reporting mortality (or equivalently the remaining living bees) is a critical task to get meaningful measures of variations in syrup consumption. For that purpose, each conditioning chamber was equipped with an IR camera sensor which automatically took pictures each hour *(Figure 2)* and additional IR light was provided in the rooms with IR LED spots for darkness pictures. Mortality was assessed following a posteriori examination of pictures and counting of dead bees visible on the conditioning chamber floor (*Figure 2*). Dead bees were removed from the chambers every day, although mortality was assessed hourly. In the dark condition, the experimenters used a flashlight covered with a red paper.

Food intake was assessed by measuring the cumulated volume dispensed by the pump each hour and divided it by the number of living bees assessed from the mortality measurement *(Figure 4B, Supplementary Figure S1)*. Being eusocial insects with a social crop, bees share food through trophallaxis; this assures all the animals within a box have approximately the same actual food storage.

2.5 Experimental protocol

To demonstrate a circadian rhythm, we measured hourly mortality and food consumption as a function of background light: constant light, constant dark, and light-dark alternation (the control group). The underlying hypothesis was that bees constantly exposed to light would lose the well-known circadian rhythm in food intake observed in constant dark and light-dark alternation, and possibly exhibit higher mortality [15,17,55,90–92].

Bees were set by cohorts of 24 or 25 animals in one of three treatments: constant dark (13 cohorts, 324 bees), constant light (12 cohorts, 299 bees), or control (i.e. 12 h light 12 h dark, light starting at 08:00; 12 cohorts, 295 bees). A cohort in the constant light treatment and a cohort in the control treatment were not used in the food intake study, so that for this analysis there were 13 cohorts in the constant dark treatment, 11 cohorts in the constant light treatment and 11 cohorts in the control. Each cohort was kept in a single box and three identical rooms without windows were used to keep all the cohorts for each of the three treatments. The treatments were administered in parallel to different cohorts for 10 complete days (starting at 0 h; the experiment and the food dispensing protocol started immediately after filling of the chambers with bees, but data recording started only from midnight). For the dark condition, no artificial light was used and the slot under and around the door were carefully masked with cardboard and tape. We also did not use the light in the adjacent corridor that could enter the rooms when opening the door. The only source of light was the low intensity OLED screens of the chambers and the control units. For that reason, as darkness was not absolute, it would be more correct to talk about "very strongly dimmed light". However, for convenience, we will continue to refer to this condition as the "dark" condition. In the light condition, the rooms were illuminated with two identical 36W, 4000 K neon tubes.

In each experimental condition, the same continuous schedule of reinforcement was active, and the reinforcer was 2 µl of sucrose solution (50% weight/weight) dispensed following each visit of the flower (8 stepper motor impulsions). This small volume has been chosen to prevent accumulation of non-consumed syrup inside the flower. The solution was replaced each day. Experiment was done at room temperature and the average temperature oscillated between 22.2 and 23.5 °C.

2.6 Data recording and processing

The data recording and the control of the experimental process was performed by a DNA propeller microcontroller board (http://1mgh.com/wiki/Dna) enclosed in a separate case from the conditioning chambers. Each control interface was able to drive and record the data from two separate chambers. The controller included a color 1.4" 4D System µOLED-128-G2 display (128 pixels * 128 pixels), a small four buttons keyboard (two per chamber) and a miniature joystick to select the

parameters of the experiment like reward amplitude (here, set at 2µl) or a fixed ratio for the reinforcement schedule (here, set at one entrance for getting one food delivery); there was no need to reprogram the microcontroller from the integrated developing environment [93]. When powered on, the controller displayed a menu with all the available options and protocols. When selected, the protocol was loaded from the controller SD card, stored inside the memory, and then started to control the experiment and record the data. During the experiment, the screen was showing the active parameters of the protocol, the number of responses, and the total amount of consumed solution.

The data were recorded in a text format and each experimental or behavioural events was recorded with a date/time information. At the end of an experimental session, the experimenter could upload the data toward a PC with a USB wire. A specific software has been written with Lazarus (https://www.lazarus-ide.org) to process the raw data, measure mortality from pictures, and get response and feeding curves per living bee *(Supplementary Figure 1)*.

2.7 Statistical analysis

Statistics were computed with R 4.3, setting the alpha risk at 5%. ~~The~~ Bee survival duration~~s~~ ~~of each bee~~ were compared across treatments using a mixed-effects Cox regression [94], using the function coxme in R. The cohorts of bees were used as a random factor ~~and~~; as not all cohorts were tested at the same time in the year, ~~the~~ day length (in minutes, computed from ~~civil~~ time for dawn and twilight) was ~~taken~~used as an additional factor, ~~.~~after confirming there was no colinearity between day duration and treatment (permutation ANOVA, p = 0.451). ~~-~~The final cumulated sucrose consumption of bees was compared across the three groups using an ANOVA followed by Scheffe post-hoc test, after taking the logarithm of the data to reach variance equality. Again, day length was added as a covariate. ~~-~~Moreover, the daily sugar consumption of bees follows a circadian rhythm, requiring a periodic modelling (possibly with a damping along the experiment, i.e. a progressive disappearance of the period). Thus, it was described for each group using a cyclic non-linear mixed model (package nlme in R). We used the following model:

$$Consumption = (A * \exp(\gamma t) + B) * \left(1 + \cos\left(2\pi \frac{t - \phi}{\tau}\right)\right) + (at + b)$$

where t is the time (in hours), γ is the damping parameter, τ is the period (expected to be around 24h for circadian rhythm) and ϕ is the phase (time of the daily peak consumption); A (size of the daily peak), B, a and b are used to adjust the equation and offset the baseline. B, a and b where random factors used to take into account measure repetition within a given BeeBox and to offset any cohort effect; they are not associated to any testable coefficient. Before modelling, data were smoothed using a moving average with a window of 3 hours (i.e. each value at t was replace by the values averaged at t-1, t and t+1). R^2 is

not defined for this type of regression. However, we computed a R^2 between the fitted values and the observed data.

3. RESULTS

For all the cohorts, it took few minutes to observe the first bee visiting the flower and receiving the syrup. Once the first bee discovered the nectar source, it took even less time for the other bees to start responding, and the overall bee responding reach its asymptotic level during the next hours (*supplementary Figure S1*).

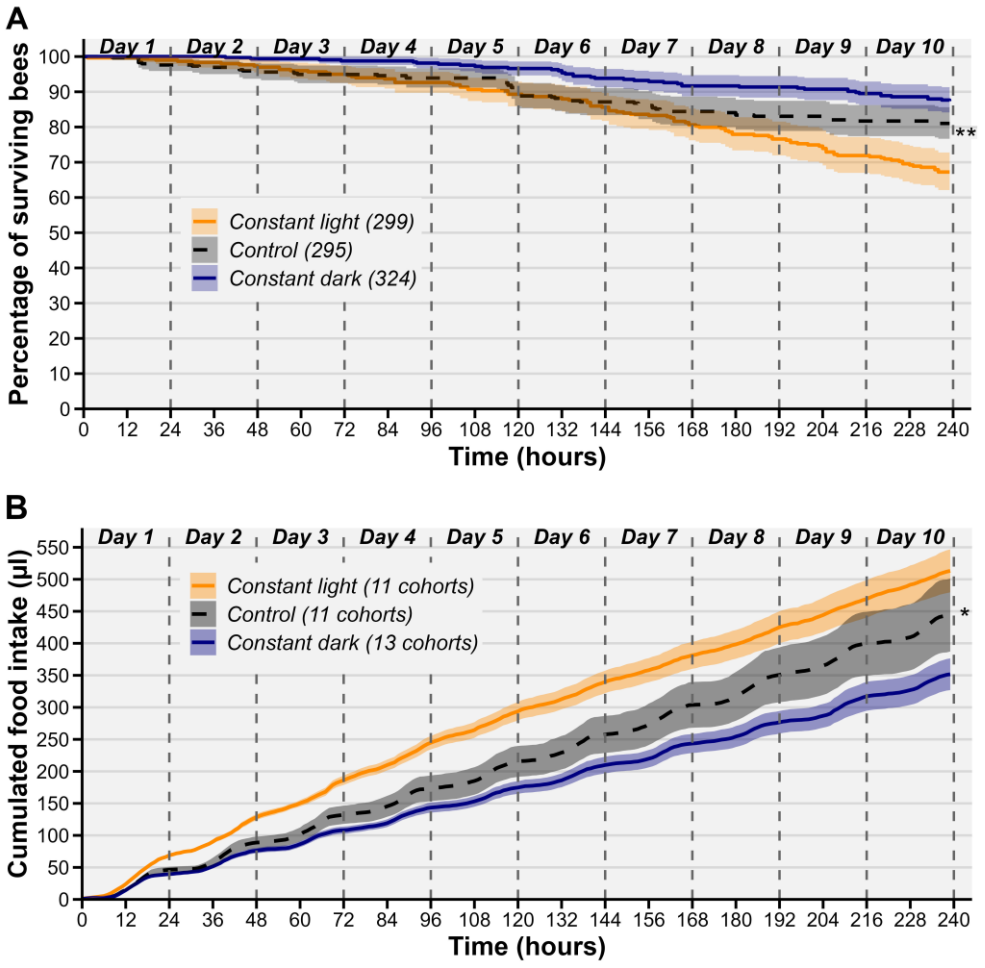

Figure 3. A. Kaplan-Meier plot for the survival of the bees as a function of time. Each curve corresponds to one of the three treatments, and the shaded area around them are their 95% confidence intervals. Numbers in parenthesis are the sample size at the beginning of the experiment. ** denotes a significant difference in the survival of bees in the ~~3~~three groups ($p < 0.010$). **B. Cumulated hourly food intake averaged per living bee.** Each curve corresponds to one of the three treatments. The shaded area around each curve is the standard error of mean. * denotes a significant difference in the total cumulated food intake in the ~~3~~~~three~~constant light and constant dark groups ($p < .05$~~5~~0).

3.1 Mortality

Bees in the control group had a 81.0% survival rate after 10 days (bootstrap-computed 95% confidence interval: 76.6%-: 85.4%). Keeping bees in constant darkness slightly improved the survival rate (87.3%, confidence interval 83.6%–: 90.7%), but the difference was not significant (Cox regression: p = 0.395, *Figure 3A*). By contrast, bees kept in constant light had a significantly lower ~~mortality~~ survival rate ~~increase~~ of ~~78~~67.2% (confidence interval: 61,9% : 72,6%, Cox regression: p = 0.004, *Figure 3A*). ~~compared to the control bees (*Figure 3A*; Cox regression, p = 0.025, value of exp(coefficient) is 1.78 and 95% confidence interval is [1.08:2.93], i.e. an increase of 8% to 193%). By contrast, bees kept in constant darkness died half often as in the control group Cox regression, p = 0.013, value of exp(coefficient) is 0.49 and 95% confidence interval is [0.28:0.86], i.e. 28% to 86%). Thus, being kept in constant light has a deleterious effect on bees' survival, whereas darkness improves it.~~Interestingly, day duration significantly decreased survival in the control group (Cox Regression: risk was 1.0056, significantly greater than 1, p = 0.004), indicating season modulate survival; bees kept in constant darkness were affected the same way (interaction in Cox Regression: p = 0.419). By contrast, this effect was compensated for bees kept in constant light (interaction in Cox Regression: risk was 0.9926, significantly lower than 1, p = 0.010), suggesting preliminary experience with daylight becomes irrelevant when bees are constantly exposed to light.

3.2 Food intake

We observed a greater food intake in animals constantly exposed to light, whereas individuals constantly kept in the dark had a lower food intake. The final cumulated food intake differed significantly between the three groups (Figure 3B; ANOVA, $F_{2, 29} = 9.598$, p = 0.0006), due to a difference between the light and the dark group (Scheffe post-hoc test, p = 0.028; for other comparisons, p ≥ 0.352). Furthermore, day duration significantly affected food intake (ANOVA, $F_{1, 29} = 45.378$, p < 0.0001); outside day duration increased food intake, indicating seasonal effect. This probably reflects indirect preliminary experience of the bees. Importantly, there was no interaction between day duration and treatment (ANOVA, $F_{2, 29} = 1.251$, p = 0.301), indicating these factors act independently. Curves also reveals daily oscillations in cumulated consumption, which suggest an ordered variation in feeding patterns that may reflect a circadian rhythm. Thus, the analysis was completed by exploring the same data but non-cumulated.~~The final cumulated food intake differed significantly between the three groups (*Figure 3B*; ANOVA on log-transformed data, $F_{2, 32} = 3.995$, p = 0.028), due to a difference between the light and the dark group (Scheffe post-hoc test, p = 0.028, for other comparisons, p ≥ 0.352). A closer look at the curves reveals daily oscillations in cumulated~~

~~consumption, which suggest an ordered variation in feeding patterns that may reflect a circadian rhythm. Thus, the analysis was completed by exploring the same data but non-cumulated.~~

3.3 Feeding patterns

Animals reared in constant light initially displayed an activity cycle with feeding occurring mainly during the initial subjective day period, but this cycle was damped after a few days (*Figure 4A* and *supplementary Figures S2* and *S3*). By contrast, control animals *(Figure 4B and supplementary Figure S2)* or animals reared in constant dark *(Figure 4C and supplementary Figure S2)* kept their initial rhythm. Hourly food intake in each group was modelled with the nlme R function; details of the model parameters are in *Supplementary Table 1*. Each of the three models yielded very significant R^2 (control, $R^2 = 0.59$; constant darkness, $R^2 = 0.58$; constant light, $R^2 = 0.36$; $p \approx 0$) and fitted the data well (dashed red line in *Figure 4*). This confirms the models were adequately describing the 8400 measures (35 cohorts for 10 days of 24 hours). The light group displayed a damping coefficient γ significantly different from 0 ($\gamma = -0.0068 \pm 0.0004$, $p < 0.001$) but not the control group ($\gamma = -0.0001 \pm 0.0002$, $p = 0.623$). The group held in constant darkness did have a modest but significant damping, but it was much less important and not evident in *Figure 4C* ($\gamma = -0.0006 \pm 0.0002$, $p = 0.009$), suggesting the absence of light only has a limited effect on food intake rhythms, whereas these are strongly affected by constant light. Consistently, the phases ϕ (peak consumption time) in control and constant darkness groups were very close (14.92 ± 0.13h and 14.78 ± 0.12h, respectively), as were their periods τ (24.17 ± 0.03h and 24.26 ± 0.02h, respectively). By contrast, as expected for the group held in constant light, both phase (15.80 ± 0.17h) and period (25.62 ± 0.07) were modified. Finally, consistent with result in *Figure 3B*, consummation peak (*A* in the model) was higher than control in the group held in constant light (3.07 ± 0.09 versus 1.63 ± 0.14), whereas in the constant darkness group it was slightly smaller (1.36 ± 0.07).

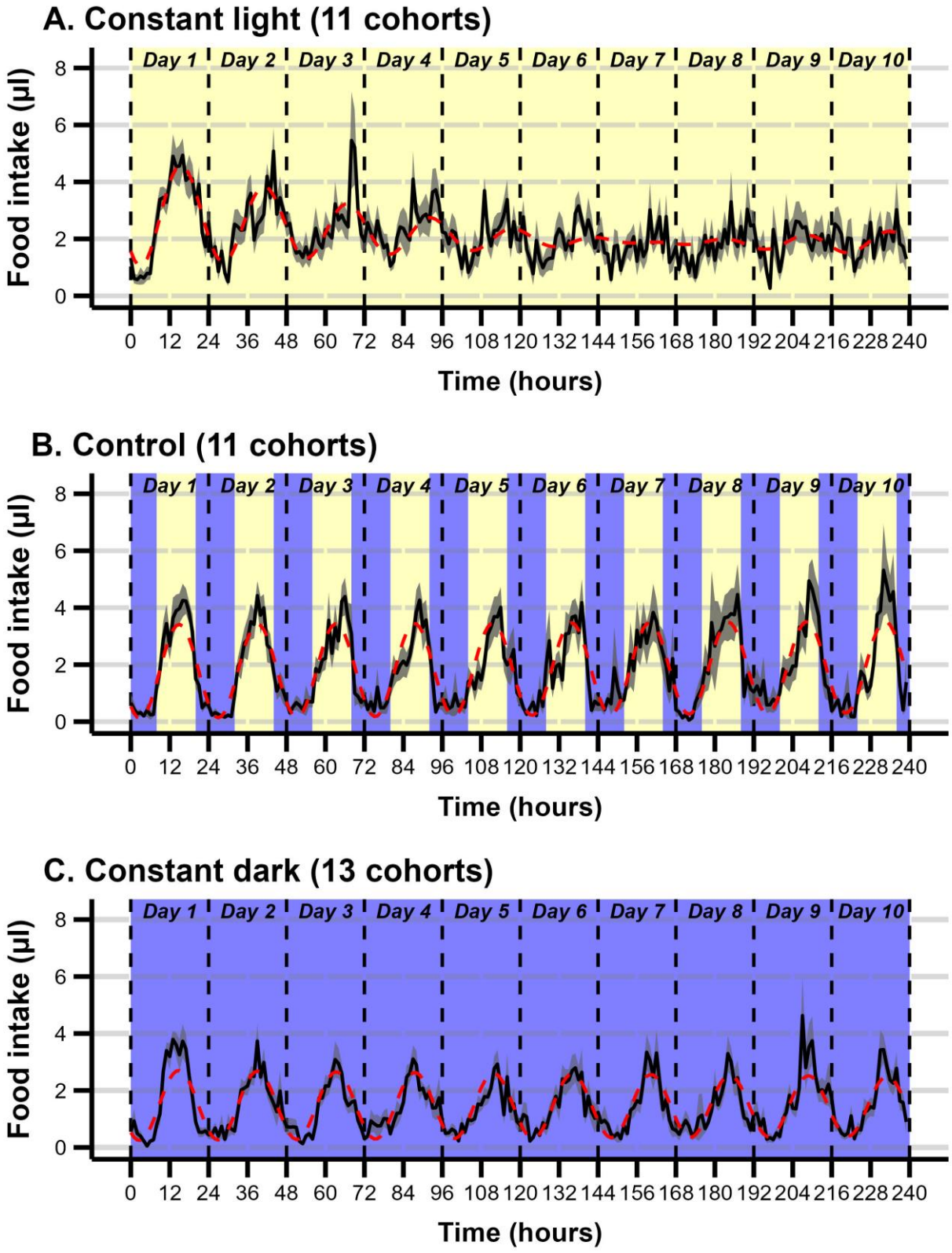

Figure 4. Hourly food intake averaged per living bee in the three conditions: constant light (A), control group (B) and constant dark (C). Data are the same as in *Figure 4B*, but not cumulated. Area in yellow or blue indicates periods of light and darkness, respectively. The shaded area around each curve is standard error of mean. Dashed red line is the fitted model. The time reported is the time elapsed since the onset of the experiment

4. DISCUSSION

Circadian rhythms of food intake in the BeeBox

In our experiments, we observed that the overall food consumption and mortality were linked to the photic environment: both were higher during the constant light condition and lower in constant darkness condition. Because we used identical rooms, experimental conditions running in parallel, and the same bee selection process, our results cannot be explained by particular bee characteristics or differential conditions; the effect of day duration at the time of each experiment was taken into account in analyses, allowing to dissociate them from the treatments. In our experiment, we measured a daily consumption between 35 and 50 µl per day per bee depending on the experimental condition. This level is comparable to the consumption observed with free available syrup dispensed with a feeder [98,99] and measured with the daily weighting of the feeder. This fact suggests that our feeding protocol (dispensing 2µl of syrup for each single flower visit) is comparable to a free feeding condition, i.e. feeding at a little of no cost [74].

Rhythmic consumption with a period longer than 24h has been observed and is largely maintained in a free run darkness test without environmental cues. To our knowledge, it is the first direct measure of circadian rhythms on food consumption in bees. Previous work has already used bees visiting feeders [18,45,49,55,56]; however, these studies did not measure food consumption but foraging intensity with locomotion or choice tests. As syrup gathered during foraging is not consumed directly but discharged and stored in the colony, syrup foraging do not reflect individual nutrition. Moreover, these studies limited the daily access to food to a short period of time. As in our protocol food was continuously available, we can exclude interpreting our results in terms of learning of time-dependent availability or as temporal control of behavior.

The level of consumption in the constant darkness condition demonstrates that even in dark, bees did not have problem to find the food source in the BeeBox. As a result, their failure to find the flower due to darkness is unlikely to explain differences between conditions. This conclusion is not surprising if we consider the relative darkness that is the rule for young bees in natural hives. Animals in constant darkness ate less and survived ~~more~~at least as much as control; they were probably less stressed. ~~The causes of this survival increase are not clear. One possible explanation could be the positive impact of li~~Limited food intake might affect~~on~~ bee longevity~~. H~~; however, while the positive impact of caloric restriction has been demonstrated in a lot of species [100], such link is still discussed in drosophila [101,102,102] and bees [103–105], for which sometimes opposing conclusions have been reached.

By contrast, in groups held in constant light the increased food consumption and mortality seem to be linked to increased locomotor activity [43–46]. Increased mortality has already been

observed in constant light in drosophila [106,107]. It is possible that mortality could result directly from phototoxic damage: the insects could die from the direct action of light [108], with no relation to rhythms or locomotion. This kind of phenomenon could contribute to the longer life expectancy of winter honey bees, which spend several months in the hive. This is also consistent with our observation that having preliminary experience with longer day durations also decreases survival (but not in the constant light group, were light exposure is saturating anyway); as we worked with young bees (which do not leave the hive), this experience might be indirect (i.e. through interactions with older, forager bees). The breakdown of the circadian rhythm could also be involved in increased mortality, although this is not the case in other insects [106]. Increased death could also occur because bees are more active possibly inducing a stress caused by lack of rest [109] or possibly because increased eating induces increased oxidative stress. However, our data does not permit to select one hypothesis and would require additional physiological investigation, which could be performed using the BeeBox.

We used young bees in our experiments; even in constant darkness young nurse bees display circadian rhythms in their locomotor activity, but they do not when they care for the brood in the presence of a light/dark cycle [110]. This indicates light has complex effects on rhythmic behaviours; what we observed with food intake is in line with their locomotor activity rather than with their brood caring, although the model did detect a modest damping. Contrary to drosophila's, hymenoptera's genome does not include the *Timeless* gene [111]. The timeless protein is destroyed by light, resulting in clock entraining. By contrast, the absence of this protein in bees prevents light to directly reset the clock; in general, in young bees, there is little oscillation of clock genes, even in regular light/dark cycle. [110]. This would explain why a rhythm in food intake can exist whether or not light is present (i.e. in control and constant darkness groups). By contrast, eExcessive light would still alter the rhythm, probably due to a phototoxic stress as discussed above. This results in a damping of the rhythm as well as a drift of the period and the phase, which our models found to be similar across the treatments. The observed damping contrasts with Fuchikawa & Shimizu [112] who did not observed damping in the same light condition. However, contrary to our study where bees where exposed to the constant light condition during ten days, Fuchikawa & Shimizu exposed bees to light during only five days after five days of dark-light alternation. It is difficult to determine whether the damping observed in our experiment would have disappeared if the experiment had been preceded by a dark-light alternation condition, or if a prolonged exposure to light would have damped Fuchikawa & Shimizu's cycles. As we used *Apis mellifera* and Fuchikawa & Shimizu worked with *Apis cerana*, it is also possible that different bee species show different rhythms patterns.

The overall damping effect could result either from a general damping observed in most bees or to a loss of synchronization between bees with a variable shift of individual phase. As social contact

is well known to activate rhythm synchronization between bees [43,50,113], the latter hypothesis is unlikely. Because all the experimental conditions were carried at the same time, and all bees from the same colony were randomly affected to these conditions, we cannot explain our results with bee characteristics or environmental variations. Bees are known to exchange food through trophallaxis [114], so that caged bees do not show a uniform food consumption [115]. Preliminary observations we did before the experiment using marked bees also showed that some bees were highly active in the flower while others were waiting food transfers. As trophallactic exchanges are common in natural colonies, food exchanges in our cages can be seen as reflecting a natural phenomenon observed in bees living in groups [116,117]. Thus, our observation of circadian rhythm on food consumption only applies at the group level. The free run test performed in the dark suggests some kind of rhythm synchronization among active bees. This kind of synchronization is not surprising in social animals and has been previously observed in numerous species including bees [50,118–121]. Yet, investigating feeding rhythms at the individual level would still be interesting; this kind of protocol would require an improved version of the BeeBox.

Here, we studied how photic-regulated circadian rhythms modulate food intake, but food itself could be a rhythm regulator. The BeeBox could also be used to study food anticipatory activities with food available only during a short period of time each day, which gives the opportunity to study the use of periodic food as a "zeitgeber". With limited period of food availability initiated at any point of the day-night cycle, timing could also be investigated, as well as fixed interval schedules of reinforcement (i.e. food is unavailable for some time after feeding) [122]. Finally, a two-flowers conditioning chamber would permit to investigate time-place learning, for example, with one flower active at one time in the day, and the other one at another time. All these protocols fit perfectly within the possibilities of the BeeBox, provided the adequate software is added to the microcontroller board.

Advantages, limitations, and potential uses of the BeeBox

In addition to making it possible to show circadian feeding patterns, the BeeBox includes several features that greatly improve the standard feeding protocol that uses caged bees with free access to artificial nectar. First, the particular shape of the sliding cover makes it possible to introduce bees without anaesthesia (*Figure 1C*). As it is known that the anaesthesia done with cold or carbon dioxide may affect honey bees [123,124], removing anaesthesia from the protocol is an interesting improvement. Second, with our device, mortality is assessed hourly, even during darkness, with automatic pictures that do not need the presence of an observer. When mortality is low, sampling rate is not critical, but with high and fast mortality following a pesticide treatment, for example, a finer resolution for mortality assessment would be useful [125]. Our device offers this possibility, and assessment time shorter than 1 hour would be possible too; we can even hypothesize a circadian

mortality in bee colonies [126]. Third, contrary to the standard bee cage device, our protocol is 100% automatized and there is no need to involve the experimenter (except when refilling food reserve). Apart during the introduction of the bees in the chamber, there is also no interaction between the experimenter and the bees. Developing automated tools that minimize the role of the experimenter is an important step toward the standardization of protocols [127] and the reproducibility of results [128]. Finally, with the same device and protocol, it is possible to simultaneously measure mortality, food consumption, and circadian rhythms in integrated experiments [44,66].

The BeeBox provides many advantages but has some limitations too. Contrary to other studies [129,130], our IR sensor detects bee entering flowers rather than proboscis extension [75]. This choice has been made to request a substantial energy expense from the bee for each response. Yet, it would be possible for some bees to activate syrup dispensing without consuming it (by entering the flower without drinking) so that the measure of consumption based on dispensed volumes would give overestimated values. To avoid this problem, we dispensed only 2µl drops of syrup. This volume has been empirically defined in such a way that it prevented syrup accumulation in the flower during successive visits.

Operant conditioning is a well-known form of learning [131,132] during which the occurrence of a behaviour is modulated by its outcome [132,133]. In the BeeBox, the trained response is flower or tube entrance while syrup acts as a reinforcer of this response. But as the syrup is given at the bottom of the tube, the reinforced response somewhat overlaps the syrup consumption response. Moreover, the stimuli associated with the operant response and with the reward are spatially combined. In pigeon, this protocol would be similar to reinforce with grain the introduction of the head inside the feeder instead of pecking a key [134]; this is also similar to nose poking conditioning in rodents [135]. Consequently, our protocol could perhaps result in a mix of sign tracking and goal tracking [136], the latter being Pavlovian conditioning rather than operant conditioning. As cues and rewards are also simultaneously presented in natural flowers, with which natur~~a~~el selection has operated, the question of how the two types of conditioning interact in natural or artificial flowers could also be explored with the BeeBox in a cognitive ecology investigation. Many protocols could also be envisioned to manipulate the syrup properties (sucrose concentration or composition) in simple flower or choice tests [68] to study the feeding strategies or nutrient choice [137,138]. Thus, the BeeBox will provide a very useful experimental set-up to perform this type of laboratory study, and also probably many others.

In addition to studies having the goal to understand the basic determinants of bee behaviour [139], our BeeBox platform could also be used in numerous applications related to several scientific fields. For example, our conditioning chambers could offer an improved alternative to the standard bee cage protocol [66,98,140] in the assessment of pesticide toxicity. Our results question the choice

of conducting such tests only in the dark conditions, as this generates the lowest level of mortality and food consumption [140]. This fact may be also problematic in chronic toxicity assessment because in darkness, the bees would consume the smallest dose of the tested pesticide at the end of the test. All this suggests that the LD50 (median lethal dose) and/or the LC50 (median lethal concentration) may be poorly measured in a number of conditions based on an arbitrary protocol choice, leading to inappropriate conclusions about the pesticide toxicity. The repeated and chronic consumption of pesticides could be investigated by adding a known concentration of pesticide in the syrup tank and running the experiment during a couple of weeks [98]. It would also be easy to define a nutritional stress [98], either by limiting reinforcer amplitude, or by increasing the amount of flower entrance required to get food (fixed-ratio reinforcement schedule); this would simulate nectar depletion of the environment.

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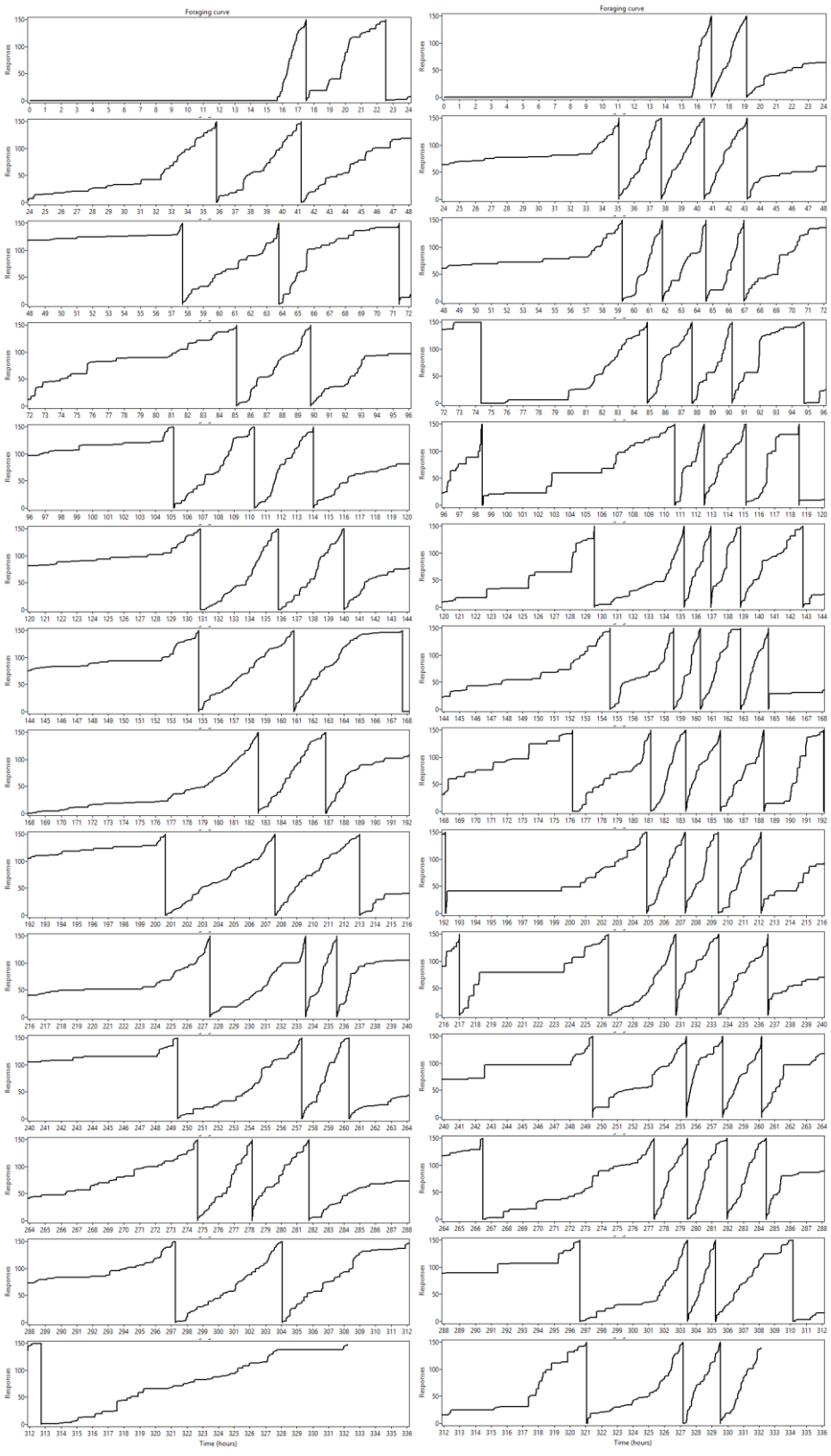
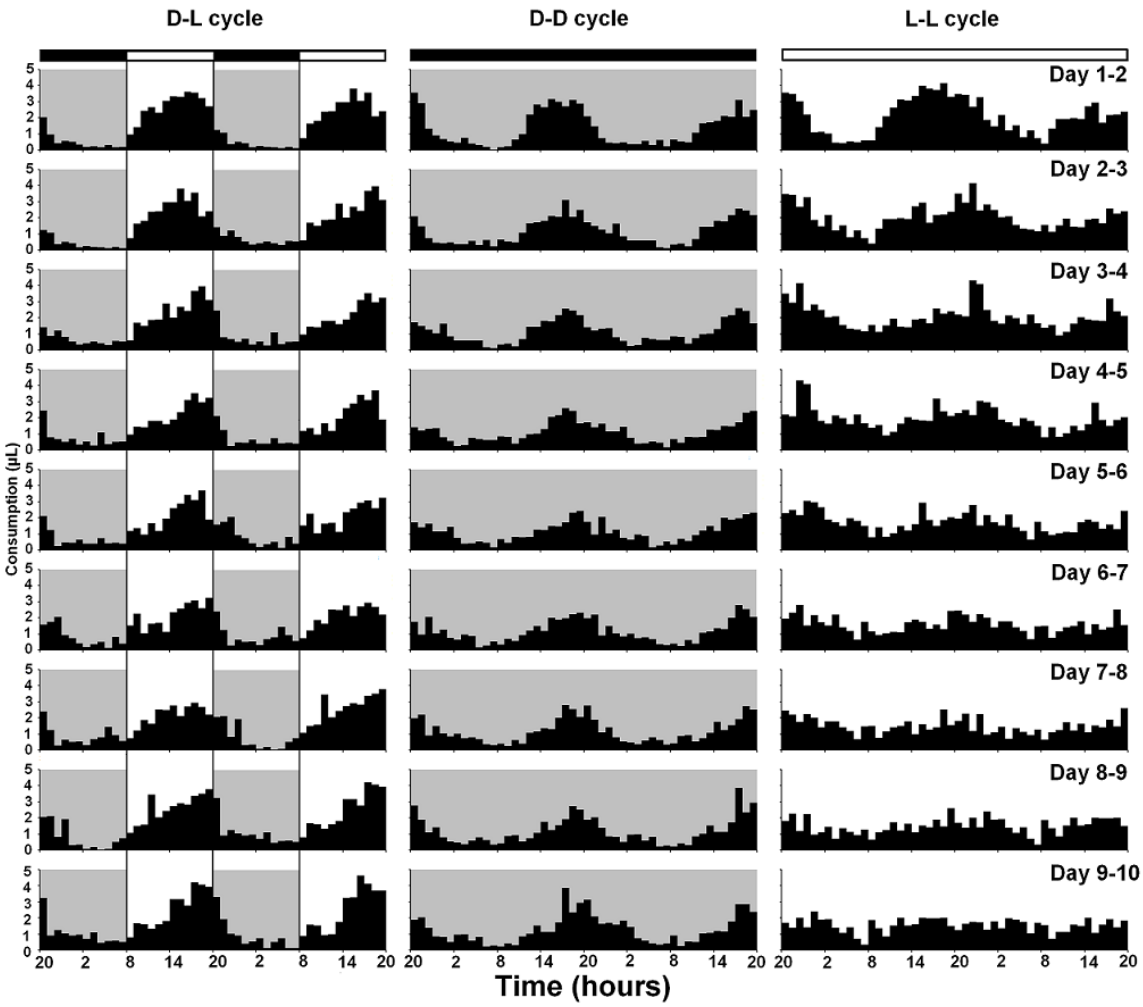
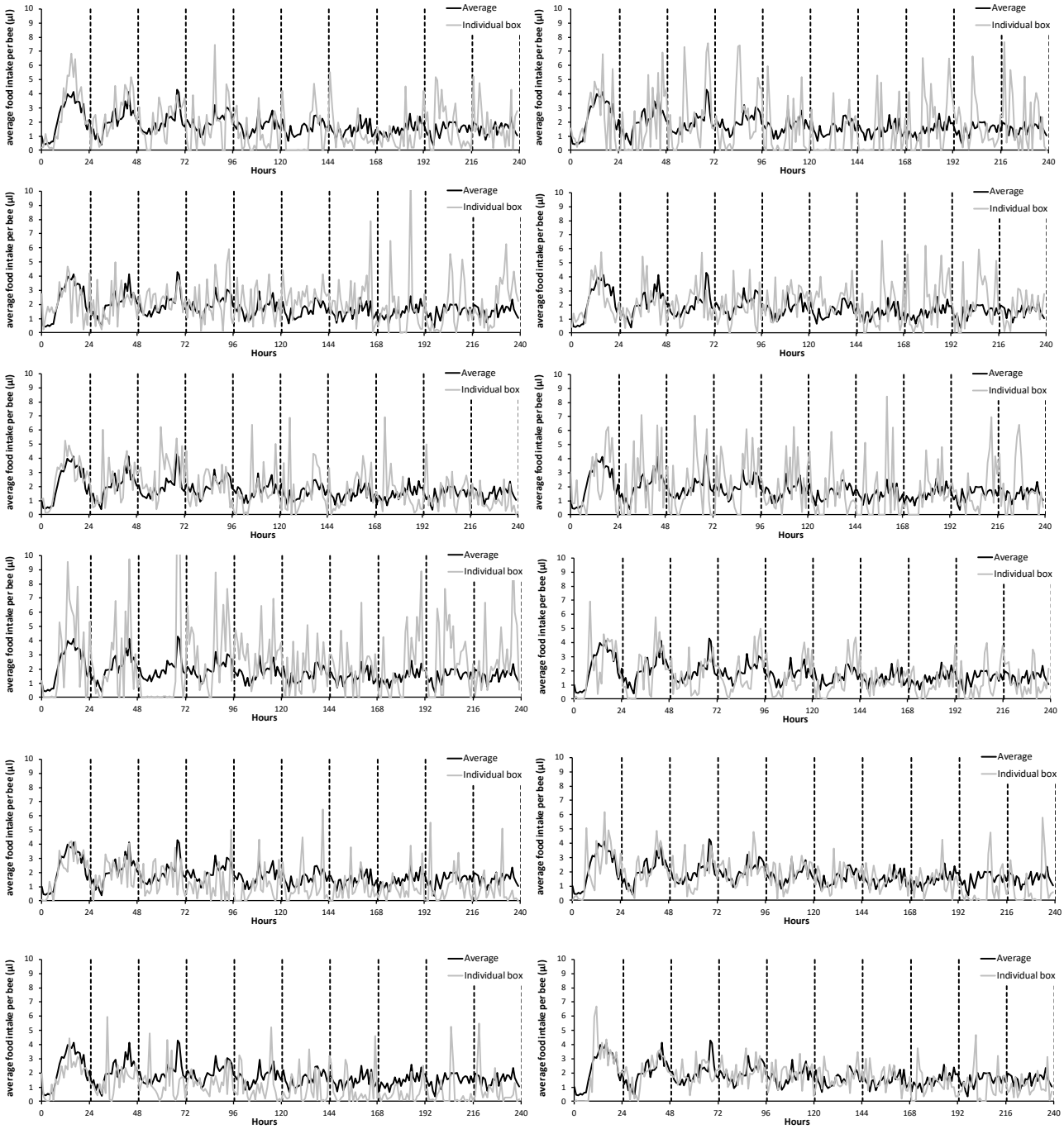


Supplementary Figure S1. Two samples (left and right) of cumulative curves for two groups of bees. Each level corresponds to 24h of experiment. The vertical axis shows cumulative responses with a reset each time the response number exceeds the vertical amplitude axis. These curves are directly drawn by the data analysis software during the experiment.



Supplementary Figure S2. Average actogram for all the conditions and bees of the experiment. The time reported is the time elapsed since the onset of the experiment. Gray areas show darkness intervals. Similar to figure 3, we can see rhythmic consumption with a maximum during the light period for the control (D-L cycle) and the always dark (D-D cycle) conditions. Damping of oscillations is detectable in the always light condition (L-L cycle).



Supplementary Figure S3. Single cohort data in the L-L condition. For comparison, the average data is also shown on each graphic. The vertical lines are separations between successive days.

Supplementary Table 1

	Estimated values	p-value
γ (damping) for control	-0.0001 ± 0.0002	0.623
γ for constant dark	-0.0006 ± 0.0002	0.009
γ for constant light	-0.0068 ± 0.0004	< 0.001
A (peak amplitude, in µl) for control	1.63 ± 0.14	< 0.001
A for constant dark	1.36 ± 0.07	< 0.001
A for constant light	3.07 ± 0.09	< 0.001
ϕ (phase, in hour) for control	14.92 ± 0.13	< 0.001
ϕ for constant dark	14.78 ± 0.12	< 0.001
ϕ for constant light	15.80 ± 0.17	< 0.001
τ (period, in hour) for control	24.17 ± 0.03	< 0.001
τ for constant dark	24.26 ± 0.02	< 0.001
τ for constant light	25.62 ± 0.07	< 0.001

The p-value test the null hypothesis that the coefficient is equal to zero. Values are provided with their standard error.

Response to reviewers

Reviewer #1: I appreciate the authors' efforts to address my concerns. Most of my concerns have been addressed properly. One unaddressed concern is how the authors averaged multiple cohorts of data conducted between April and June not being clearly stated. Specifically, in Figure 4 A and C, Figure S2 D-D and LL, what actual times do the time of day (y-axis) represent? Obviously, data is plotted relative to the LD cycle for the LD group, but how do the authors adjust time in multiple cohorts of experiments done under different day lengths? Was the middle of the day centered, plotted relative to sunrise time or relative to local time (if so how was daylight saving time adjusted)?

When checking the data a last time, the first author realized he did a mistake when reporting when the experiment took place. The initial statement was that the experiment took place between April and June. The correct information is “Experiment took place between April and June 2016 and later between end of September-beginning of October”. The first author sincerely apologizes for his error.

In the Figures, the time reported is the time elapsed since the onset of the experiment. As stated in the article, recording started at midnight (0h00) in all cases, so that all the plots are aligned with this starting point. We understand the reviewer is concerned by the day light duration outside of the laboratory, which is dependent upon the time of the year at which the experiments were performed. We did not adjust the time on the plot to reflect this, as the point of the plot is to compare aligned data, and because what an appropriate adjustment should be is not clear anyway. Rather, we complemented our analysis by adding as a factor the day duration (in minutes) at the time of bee collection, as obtain from Almanac for computer 1990 (Nautical almanac office, United State naval observatory, Washington D). This revealed new effect complementing what we already reported, so this was definitely a good suggestion. The text was modified accordingly (2.7 Statistical analysis; 3.1 Mortality results; 3.2 food intake results; 4. Discussion - Circadian rhythms of food intake in the BeeBox); however, the plots were left unchanged, as this is not the point of the article and the experiments were not designed for completely exploring this factor.

P5 line 42-43, Clock (italic) should be clock (italic) as insect genes normally use lowercase with italics.

Done

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HIGHLIGHTS

- The BeeBox automatically records feeding time and occurrence in insects.
- This reveals circadian rhythm in feeding in bees.
- This rhythm is lost in animals kept in constant light.
- Constant light also induces higher mortality.

Measuring honey bee feeding rhythms with the Beebox, a platform for nectar foraging insects

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Declarations of interest: none

HIGHLIGHTS

- The BeeBox automatically records feeding time and occurrence in insects.
- This reveals circadian rhythm in feeding in bees.
- This rhythm is lost in animals kept in constant light.
- Constant light also induces higher mortality.

ABSTRACT

In honey bees, most studies of circadian rhythms involve a locomotion test performed in a small tube, a tunnel, or at the hive entrance. However, despite feeding playing an important role in honey bee health or fitness, no demonstration of circadian rhythm on feeding has been performed until recently. Here, we present the BeeBox, a new laboratory platform for bees based on the concept of the Skinner box, which dispenses discrete controlled amounts of food (sucrose syrup) following entrance into an artificial flower. We compared caged groups of bees in 12h-12h light/dark cycles, constant darkness and constant light and measured average hourly syrup consumption per living bee. Food intake was higher in constant light and lower in constant darkness; mortality increased in constant light. We observed rhythmic consumption with a period longer than 24h; this is maintained in darkness without environmental cues, but is damped in the constant light condition. The BeeBox offers many new research perspectives and numerous potential applications in the study of nectar foraging animals.

Key-words: honey bees, Skinner box, feeding patterns, circadian rhythms, mortality assessment, operant conditioning

1. INTRODUCTION

As many other species, honeybees are diurnal animals whose behavior and physiology are strongly affected by the cyclic dimension of their physical and biological environment. In many latitudes, the cyclic alternation of day and night has a strong impact on flower resources gathered by bees (mainly nectar and pollen), and flowers activity shows some kind of circadian rhythmicity [1]. For numerous flowers, nectar production and nectar sugar content depend on the time of the day [2–8] with a peak observed in the morning for some flowers [9,10] and in the afternoon for others [11,12]. Moreover, some flowers also show opening and closing cycles [13] that limit the availability of nectar and pollen. When evolution takes place in such an environment involving plant-pollinator interactions and co-adaptation, temporal match between honeybees and flower traits can be expected [14–16]. Consequently, it is not surprising observing bees visiting flowers when nectar is at its highest level [12,17,18]. Two kinds of behavioral phenomena can be potentially involved in this kind of temporal match: spontaneous circadian rhythms and learning [19–21].

Circadian rhythms refer to an endogenous oscillation in a biological variable that last approximately 24 h [19,22,23]. Such a rhythm is not learned, but entrained by some environmental signal, also called "zeitgeber" [24], that can be, for example, light, temperature, or food availability [19,25]. Entrainment refers to an active mechanism that synchronizes the biological variable to the cyclic occurrence of the "zeitgeber" [26]. It is often assumed that such rhythms may procure a selective advantage with the anticipation of some predictable and cyclic environmental important events [15,16] such as availability of flower resources for honeybees.

However, circadian rhythms are not the only mechanisms to adjust to time-dependent events. Learning allows anticipating periodic events, most often food [27]. Traditionally, temporal regulation has been studied by psychologists with pavlovian [28] or operant conditioning timing protocols [29], for example with single food items being presented at regular time intervals independently, or as a consequence, of some arbitrary response. Interval timing or temporal conditioning occurs if the animal anticipates the occurrence of the food event with no other cue than the time since the last food occurrence [30,31]. As demonstrated in vertebrates, circadian rhythms and temporal conditioning may involve a variety of physiological mechanisms [19,32,33] and may interact together [34]. Classic temporal conditioning involves seconds to minute intervals with time intervals initiated at any point of the day-night cycle [30] when circadian rhythm studies concern 24 h duration intervals. However, it has also been suggested that temporal regulation may happen over longer intervals in food-anticipatory activities [30,35]. In these studies, the protocol combines a circadian and a timing dimension: food is available only at a fixed point of the day-night cycle [30,36] and then removed. Such protocols differ from pure interval timing studies because food is not presented after a given

delay but is only available for 2-4 hours [37]. The task may be a little bit more complex if the protocol includes also a spatial dimension, in a so-called time-place learning protocol where the location of a food item or a meal depends on the time of the day [38,39], with one or more, locations available [40,41].

When food is presented periodically with a period of 24h, learning and circadian rhythms are two potential candidates to explain periodic responding. In order to be a relevant adjustment to environmental conditions, a circadian rhythm must be entrained by food occurrence, which then act as a "zeitgeber"[42]. If animals develop temporally conditioned or food-anticipatory responses, then food may become an anticipated unconditioned stimulus [37,38]. Both mechanisms could act alone, or combine their effects. [39].

In bees, most studies on circadian rhythms involved a locomotion test. Such test is most often performed with a bee encaged in a small tube or a tunnel equipped with infrared sensors [43–47], performed with video tracking [48], or at the colony entrance [49–51]. Yet, locomotion is only an indirect measure of food gathering; rather, honeybee health and colony fitness are directly related to nectar consumption [52,53]. Unfortunately, while circadian rhythms in locomotion have been studied, very few tests have studied feeding rhythms [54]. Feeding opportunity has been sometimes manipulated in food-anticipatory or time-place learning protocols [18,45,49,55,56] but food consumption was not measured, and studying foraging rhythms with bees is a quite different problem than studying individual food consumption because, nectar gathering during foraging activity does not reflect metabolic activity.

Yet, such links between feeding and rhythms seems to exist: for instance, female (but not male) flesh flies *Sarcophaga crassipalpis* exhibit an extended scotophase activity according to their nutrition (and their age), suggesting food intake can modulate circadian rhythm [57]. Similarly, food deprivation can alter sleeping patterns [58]. Such effects can be explained by an impact of food intakes on the gene expression balance that finely regulates circadian rhythms [59–61], such as *clock*, *period* and *timeless*. Indeed, in the sandfly *Lutzomyia longipalpis*, a blood meal can downregulate period and timeless gene expression levels [59]. Moreover, food intake during foraging can lead to pesticide contamination (especially in pollinators like bees), which is known to affect rhythms too [62,63].

Overall, these results point to the need for more studies on the rhythm of food intake in insects, or on the role of food in rhythm entrainment, especially in bees. The lack of feeding tests in honey bee rhythm research could reflect the lack of a proper technology to measure consumption in long periods. A protocol commonly used in feeding experiments involves small cages with nearly-unlimited and easily accessible food, most often syrup (concentrated sucrose solution) stored in a feeder [64–66]. Mortality and food consumption are assessed punctually, most often daily, by

respectively manual counting of dead bees and weighing the feeder [67,68] or measuring the level of food inside a tube [69]. However, such manual protocols do not allow fine studying of circadian rhythms because there is the need to measure consumption repeatedly (i.e. hourly) during several consecutive days. Automated feeding-measurement protocols have become popular in others species and several approaches have been developed to assess circadian feeding rhythms and record consumption repeatedly during the long term [70]. One of them involves a Skinner box, where food is delivered in small quantity provided animals release a specific response [71–73]. With such a device, the animal has to emit repeatedly the response to get its daily food ration [74]. Unfortunately, while already used for short duration free flying experiments with bees [75], or lab experiments with flies [76], previously used tools are inadequate in laboratory setting.

To fill this gap, we recently developed a new concept of laboratory Skinner box [54,77] able to deliver small controlled amounts of syrup to bees visiting an artificial flower. The box allows precise measures of food intake time and occurrence during several days. This paper follows three main objectives. First, we present our new automatized conditioning chamber, the "BeeBox". Second, using this new tool and a "true" circadian protocol with continuous access to food, we explore feeding rhythms in honeybees and whether these rhythms can be modulated by light conditions. Finally, we discuss the potential applications of our platform to deepen the study of circadian rhythms and to explore other fields related to ecological, behavioral, physiological and ecotoxicological research in nectar foraging animals.

2. MATERIAL AND METHOD

2.1 Animals

Experiment took place between April and June 2016 and later between end of September-beginning of October at the Université de Picardie – Jules Verne, Amiens (France). Honey bees (Buckfast) were caught on one frame of a single 10-frames Dadant hive located in the apiary close to the laboratory early in the afternoon. The frame was extracted from the hive and then kept vertical on a table with the help of a wooden support. Bees were then collectively captured with 30ml sample plastic containers and quickly introduced in the BeeBox without anaesthesia with the help of the sliding cover (see apparatus section). Each BeeBox was then randomly assigned to one of the three treatments (see below). This procedure was preferred to catching leaving or returning foragers for two reasons. First, foragers are known to be older bees. To illustrate the interest of the BeeBox for long-term measurements, our experiment was planned for at least 10 days; thus, we wanted to limit natural mortality due to age, leading us to avoid foragers. Second, we had to fill chambers with several hundreds of bees and had only a short time to perform this task. The collective capture of bees with

several 30 ml plastic containers took only few minutes and it would have taken much more time to catch foragers one by one at the hive entrance. This protocol does not control the exact age of the bees, but increases the probability to work with young bees; it also minimizes handling and stress, and avoids anaesthesia. Furthermore, bees are randomly assigned to each treatment. We did not capture drones and bees with pollen balls because drones have a larger size that do not fit to the size of the flower diameter and pollen balls could be stored inside the response hole and block the dispensing needle (see *Figure 1. A*); moreover, pollen balls are carried by forager bees. Studying foragers would definitely be important, as it is known forager and non-foragers bees have differing circadian rhythms [78].

2.2 Apparatus

The 12 identical BeeBox (overall dimensions: 20cm x 12cm x 25cm, L x l x h) were composed of three parts *(Figure 1 and Figure 2).*

The bottom part included the printed circuit board, a peristaltic pump (6 rollers Kamoer KCS stepper motor peristaltic pump), a small OLED display (Adafruit Monochrome 0.96" 128x64 OLED graphic display, https://www.adafruit.com/product/326), a three push button keyboard, the on/off button, and a 40mm x 40mm fan.

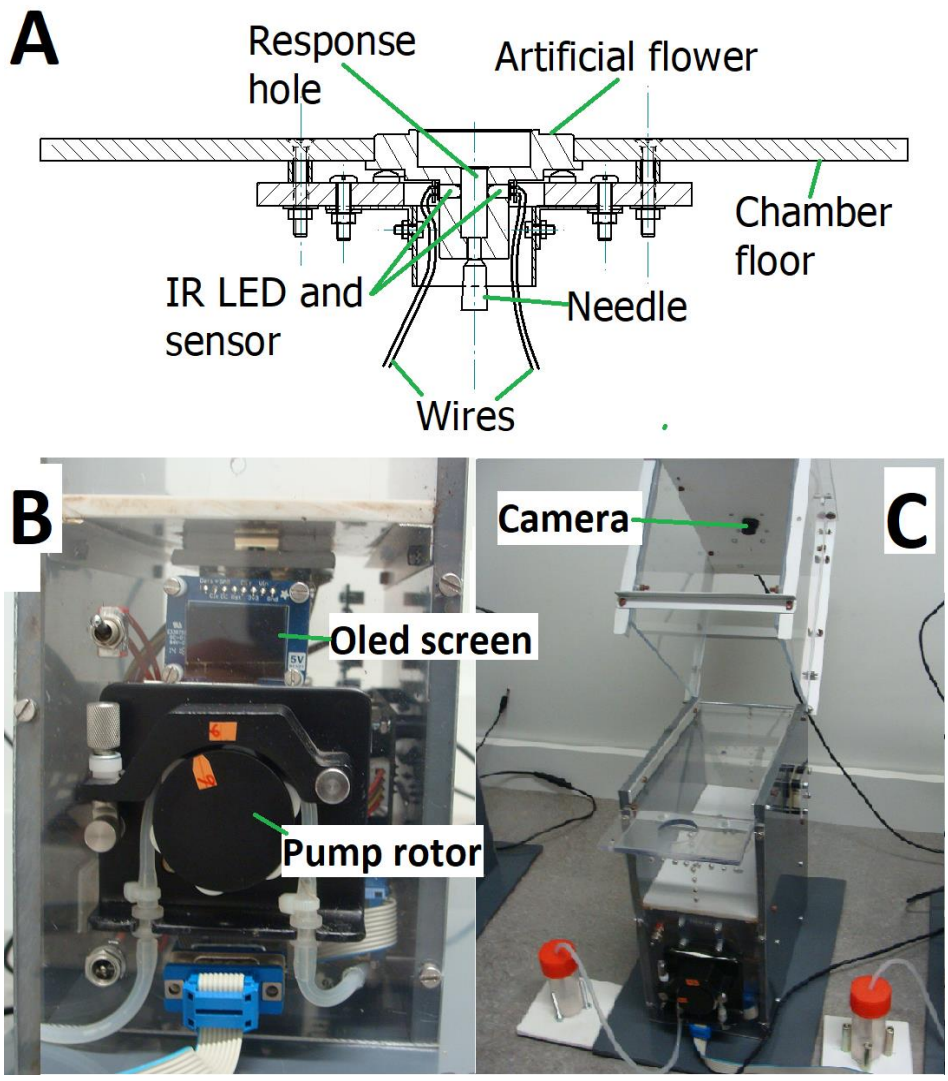

Figure 1. A. Drawings of a sectional view of the artificial flower fixed on the floor of the bee chamber. The IR LED sensors are connected to the pump controller. If needed, it is possible to add a 16 RGB LED ring under the flower to color it from below. The silicone tubing connected to the needle is not shown. **B.** Front view of the bottom part of the conditioning box showing the pump and the silicone tubing. Orange labels are used to set the reference position. **C**. BeeBox with the top camera part lifted. After being captured in tubes in the hive, bees are set in this central part without anesthesia by fitting the tube in a circular hole in the top of the box, and then sliding it over the box to release them. The syrup (sucrose solution used to feed the bees) is stored in the red cap 30ml plastic bottle.

The center part is the cage for the bees (20cm * 10cm * 9cm height). It had 32 3mm holes for ventilation and a transparent sliding cover. This cover allows to introduce awake bees from a standard 30ml sample container without any risk of escape. The front and back face were in transparent polycarbonate for visual observation, and the side faces in opaque PVC. The floor of the bee cage was removable for cleaning. The main part of the conditioning chamber was a 46 mm diameter cylindrical polyamide artificial flower machined with a lathe and screwed in the centre of the floor. In the centre of the flower, we drilled a 6mm diameter/17 mm long hole (i.e. approximate size of a bee) with a standard 25G Luer needle (needle cut flush to the cone) glued in the bottom for syrup dispensing. The surface of the flower contained a small 2mm high rim serving as an obstacle to prevent any dead bee from falling inside the flower opening.

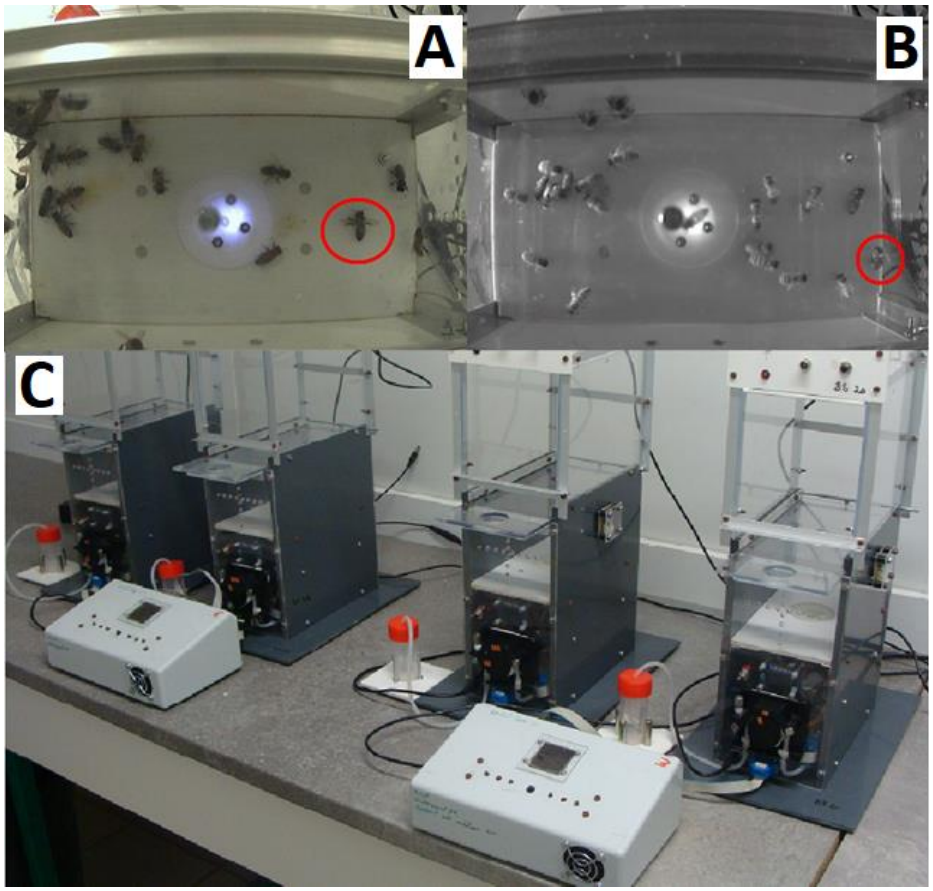

Figure 2. A. Sample picture for mortality assessment during the light-ON condition**. B.** Sample picture for mortality assessment during the dark condition. In each case, a dead bee (circled with red) can be identified. The bright spot visible in the center of the flower (panel A and panel B) corresponds to light from the IR LED. **C.** Picture of the experimental setup for each of the three rooms (four BeeBoxes in each). Each control interface drives two separate conditioning chambers.

The top part of the BeeBox is a swivel cover (20cm * 12cm * 21cm) equipped with a sensor camera (PTC08 serial JPEG camera with NTSC video) for mortality measurement. The serial camera was driven by an Arduino Uno board improved with a real time clock and a SD card reader (Adafruit Data Logging shield for Arduino) and programmed to take a picture each hour and to store it on the SD card. The devices were powered with a set of six standard 12V DC power modules that could power each two different conditioning chambers.

To get food, a honey bee had to enter in a vertical hole in the centre of the artificial flower; this entrance was detected with an IR Sensor (*Figure 1A*), which in turn triggered the release of food in a smooth and consistent way. These features correspond to the hallmarks of a Skinner box [75,79]: a well-defined behaviour (i.e., entrance in the tube) and a resource contingent to the production of this behaviour (food syrup, which is the reinforcement in the terminology of Skinner box protocol). It is also possible to add stimuli signalling the availability of the resource, such as light: a 16 RGB LED ring can be placed under the flower so that it is also possible to colour the flower from below and diffuse light into the polyamide disc. This allows to condition the food release to the presence of the appropriate visual background, but this was not used in the experiments reported here. Similarly, food could have been offered only during specific time [12,17,18], although this possibility was not used either in this work. The number of entrances required to release the food, as well as the dispensed amount of syrup, can also be controlled. This allows to control the effort required to obtain food; here, a single entrance was required. The BeeBox permits to continuously monitor food intake and mortality and controls the effort required to obtain food (i.e. the number of entrance) in a standardized way with reinforcement schedules, thus avoiding the need of experimenter's intervention. The approximate cost are 250€ for the conditioning chamber, 150€ for the top camera part and 250€ for the control interface, which gives a total of 650 €.

2.3 Syrup dispensing

A major technical problem with delivering small amount of liquid food with a peristaltic pump is the pulsation that is characteristic to this kind of pump because they use several rollers pressing a silicone tubing. Each time a roller takes off from the tube, reverse pumping occurs, and the pump dispenses nothing. Depending on the pump, the "no distribution" phase of the pump can vary until 30% to 45% of the total rotary cycle. Such problem prevents using this kind of pump to deliver with precision small discrete volumes of liquids (less than 1 µl). To solve it, we set a compensatory algorithm in the BeeBox controller working with stepper motor pumps. To measure and control the dispensed volume, we count the impulsions that are sent to the motor without counting impulsions when reverse pumping occurs [80]. Thus, initially the experiment pumps (Figure 1B) were manually

set to a reference angular position. The compensation amplitude was then measured empirically and depends on the pump and the tube properties. We used six rollers miniature pumps driven by a 200 steps motor with 0.8mm internal diameter silicone tubing, and a P8X32A microcontroller to drive the pump, monitor the angular position and compensate the "no distribution" phase. With one stepper motor step, the pumps dispense about 0.25µl [81,82]. However, larger volumes can be obtained with the successive activation of the stepper motor.

Our compensation algorithm paves the way for more applications using inexpensive peristaltic pumps to dispense small volumes of liquids and our device could be adapted to other species of nectar foraging animals like bumblebees [83], butterflies [84], moths [77,85], ants [86], or hummingbirds [87,88]. Moreover, even if the algorithm has been designed to dispense discrete amounts of syrup, the repeated activation of the stepper motor activated pump could also control low syrup flow rates [89].

To increase sample size in our experiment, we used cohorts of bees rather than single individuals. Testing individual bees would have generated a very low syrup flow with a risk of clogging the dispensing nozzle with dried syrup. Testing individual bees would need additional technical options to prevent this.

2.4 Mortality and food intake measurement

As there are several bees in each box, reporting mortality (or equivalently the remaining living bees) is a critical task to get meaningful measures of variations in syrup consumption. For that purpose, each conditioning chamber was equipped with an IR camera sensor which automatically took pictures each hour *(Figure 2)* and additional IR light was provided in the rooms with IR LED spots for darkness pictures. Mortality was assessed following a posteriori examination of pictures and counting of dead bees visible on the conditioning chamber floor (*Figure 2*). Dead bees were removed from the chambers every day, although mortality was assessed hourly. In the dark condition, the experimenters used a flashlight covered with a red paper.

Food intake was assessed by measuring the cumulated volume dispensed by the pump each hour and divided it by the number of living bees assessed from the mortality measurement *(Figure 4B, Supplementary Figure S1)*. Being eusocial insects with a social crop, bees share food through trophallaxis; this assures all the animals within a box have approximately the same actual food storage.

2.5 Experimental protocol

To demonstrate a circadian rhythm, we measured hourly mortality and food consumption as a function of background light: constant light, constant dark, and light-dark alternation (the control group). The underlying hypothesis was that bees constantly exposed to light would lose the well-known circadian rhythm in food intake observed in constant dark and light-dark alternation, and possibly exhibit higher mortality [15,17,55,90–92].

Bees were set by cohorts of 24 or 25 animals in one of three treatments: constant dark (13 cohorts, 324 bees), constant light (12 cohorts, 299 bees), or control (i.e. 12 h light 12 h dark, light starting at 08:00; 12 cohorts, 295 bees). A cohort in the constant light treatment and a cohort in the control treatment were not used in the food intake study, so that for this analysis there were 13 cohorts in the constant dark treatment, 11 cohorts in the constant light treatment and 11 cohorts in the control. Each cohort was kept in a single box and three identical rooms without windows were used to keep all the cohorts for each of the three treatments. The treatments were administered in parallel to different cohorts for 10 complete days (starting at 0 h; the experiment and the food dispensing protocol started immediately after filling of the chambers with bees, but data recording started only from midnight). For the dark condition, no artificial light was used and the slot under and around the door were carefully masked with cardboard and tape. We also did not use the light in the adjacent corridor that could enter the rooms when opening the door. The only source of light was the low intensity OLED screens of the chambers and the control units. For that reason, as darkness was not absolute, it would be more correct to talk about "very strongly dimmed light". However, for convenience, we will continue to refer to this condition as the "dark" condition. In the light condition, the rooms were illuminated with two identical 36W, 4000 K neon tubes.

In each experimental condition, the same continuous schedule of reinforcement was active, and the reinforcer was 2 µl of sucrose solution (50% weight/weight) dispensed following each visit of the flower (8 stepper motor impulsions). This small volume has been chosen to prevent accumulation of non-consumed syrup inside the flower. The solution was replaced each day. Experiment was done at room temperature and the average temperature oscillated between 22.2 and 23.5 °C.

2.6 Data recording and processing

The data recording and the control of the experimental process was performed by a DNA propeller microcontroller board (http://1mgh.com/wiki/Dna) enclosed in a separate case from the conditioning chambers. Each control interface was able to drive and record the data from two separate chambers. The controller included a color 1.4" 4D System µOLED-128-G2 display (128 pixels * 128 pixels), a small four buttons keyboard (two per chamber) and a miniature joystick to select the

parameters of the experiment like reward amplitude (here, set at 2µl) or a fixed ratio for the reinforcement schedule (here, set at one entrance for getting one food delivery); there was no need to reprogram the microcontroller from the integrated developing environment [93]. When powered on, the controller displayed a menu with all the available options and protocols. When selected, the protocol was loaded from the controller SD card, stored inside the memory, and then started to control the experiment and record the data. During the experiment, the screen was showing the active parameters of the protocol, the number of responses, and the total amount of consumed solution.

The data were recorded in a text format and each experimental or behavioural events was recorded with a date/time information. At the end of an experimental session, the experimenter could upload the data toward a PC with a USB wire. A specific software has been written with Lazarus (https://www.lazarus-ide.org) to process the raw data, measure mortality from pictures, and get response and feeding curves per living bee *(Supplementary Figure 1)*.

2.7 Statistical analysis

Statistics were computed with R 4.3, setting the alpha risk at 5%. Bee survival durations were compared across treatments using a mixed-effects Cox regression [94], using the function coxme in R. The cohorts of bees were used as a random factor; as not all cohorts were tested at the same time in the year, day length (in minutes, computed from time for dawn and twilight) was used as an additional factor, after confirming there was no colinearity between day duration and treatment (permutation ANOVA, p = 0.451). The final cumulated sucrose consumption of bees was compared across the three groups using an ANOVA followed by Scheffe post-hoc test, after taking the logarithm of the data to reach variance equality. Again, day length was added as a covariate. Moreover, the daily sugar consumption of bees follows a circadian rhythm, requiring a periodic modelling (possibly with a damping along the experiment, i.e. a progressive disappearance of the period). Thus, it was described for each group using a cyclic non-linear mixed model (package nlme in R). We used the following model:

$$Consumption = (A * \exp(\gamma t) + B) * \left(1 + \cos\left(2\pi \frac{t - \phi}{\tau}\right)\right) + (at + b)$$

where t is the time (in hours), γ is the damping parameter, τ is the period (expected to be around 24h for circadian rhythm) and ϕ is the phase (time of the daily peak consumption); A (size of the daily peak), B, a and b are used to adjust the equation and offset the baseline. B, a and b where random factors used to take into account measure repetition within a given BeeBox and to offset any cohort effect; they are not associated to any testable coefficient. Before modelling, data were smoothed using a moving average with a window of 3 hours (i.e. each value at t was replace by the values averaged at t-1, t and t+1). R^2 is not defined for this type of regression. However, we computed a R^2 between the fitted values and the observed data.

3. RESULTS

For all the cohorts, it took few minutes to observe the first bee visiting the flower and receiving the syrup. Once the first bee discovered the nectar source, it took even less time for the other bees to start responding, and the overall bee responding reach its asymptotic level during the next hours (*supplementary Figure S1*).

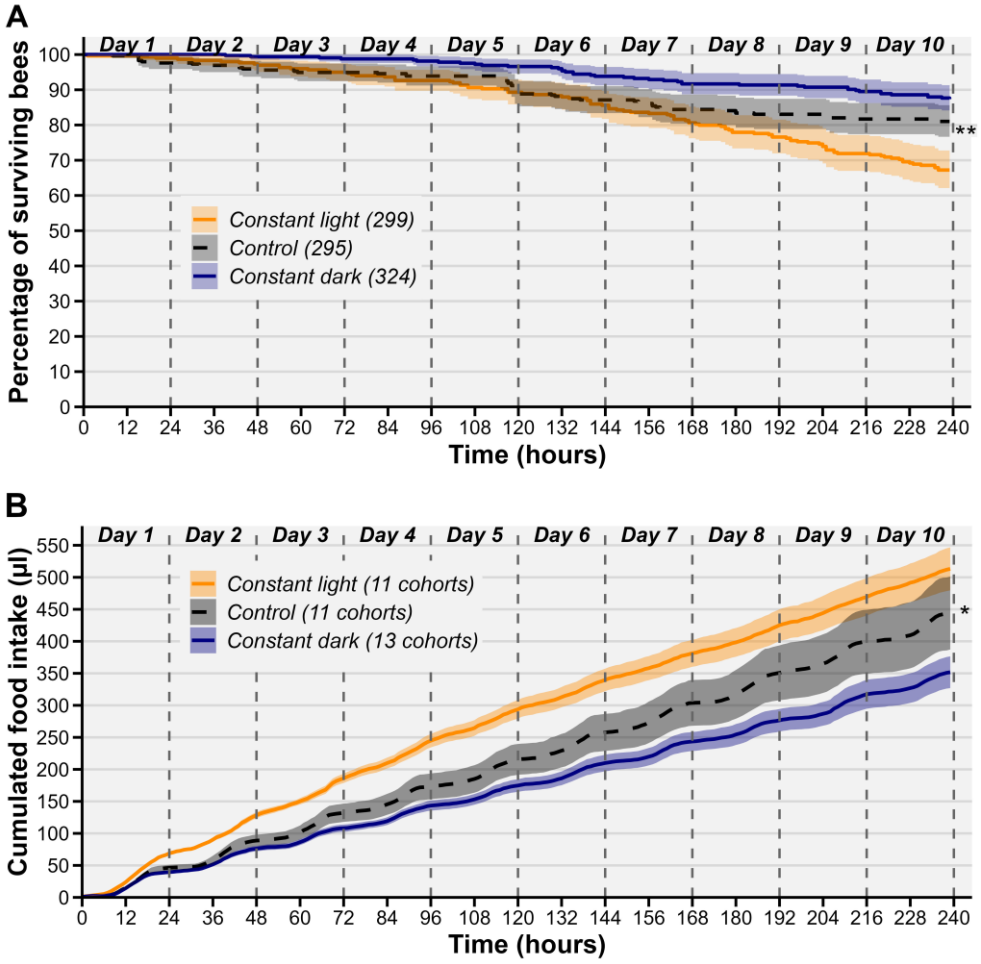

Figure 3. A. Kaplan-Meier plot for the survival of the bees as a function of time. Each curve corresponds to one of the three treatments, and the shaded area around them are their 95% confidence intervals. Numbers in parenthesis are the sample size at the beginning of the experiment. ** denotes a significant difference in the survival of bees in the three groups ($p < 0.010$). **B. Cumulated hourly food intake averaged per living bee.** Each curve corresponds to one of the three treatments. The shaded area around each curve is the standard error of mean. * denotes a significant difference in the total cumulated food intake in the constant light and constant dark groups ($p < .050$).

3.1 Mortality

Bees in the control group had a 81.0% survival rate after 10 days (bootstrap-computed 95% confidence interval: 76.6%: 85.4%). Keeping bees in constant darkness slightly improved the survival rate (87.3%, confidence interval 83.6%: 90.7%), but the difference was not significant (Cox regression: $p = 0.395$, *Figure 3A*). By contrast, bees kept in constant light had a significantly lower survival rate of 67.2% (confidence interval: 61,9% : 72,6%, Cox regression: $p = 0.004$, *Figure 3A*). Interestingly, day duration significantly decreased survival in the control group (Cox Regression: risk was 1.0056, significantly greater than 1, $p = 0.004$), indicating season modulate survival; bees kept in constant darkness were affected the same way (interaction in Cox Regression: $p = 0.419$). By contrast, this effect was compensated for bees kept in constant light (interaction in Cox Regression: risk was 0.9926, significantly lower than 1, $p = 0.010$), suggesting preliminary experience with daylight becomes irrelevant when bees are constantly exposed to light.

3.2 Food intake

We observed a greater food intake in animals constantly exposed to light, whereas individuals constantly kept in the dark had a lower food intake. The final cumulated food intake differed significantly between the three groups (Figure 3B; ANOVA, $F_{2, 29} = 9.598$, $p = 0.0006$), due to a difference between the light and the dark group (Scheffe post-hoc test, $p = 0.028$; for other comparisons, $p \geq 0.352$). Furthermore, day duration significantly affected food intake (ANOVA, $F_{1, 29} = 45.378$, $p < 0.0001$); outside day duration increased food intake, indicating seasonal effect. This probably reflects indirect preliminary experience of the bees. Importantly, there was no interaction between day duration and treatment (ANOVA, $F_{2, 29} = 1.251$, $p = 0.301$), indicating these factors act independently. Curves also reveals daily oscillations in cumulated consumption, which suggest an ordered variation in feeding patterns that may reflect a circadian rhythm. Thus, the analysis was completed by exploring the same data but non-cumulated.

3.3 Feeding patterns

Animals reared in constant light initially displayed an activity cycle with feeding occurring mainly during the initial subjective day period, but this cycle was damped after a few days (*Figure 4A* and *supplementary Figures S2* and *S3*). By contrast, control animals *(Figure 4B and supplementary Figure S2)* or animals reared in constant dark *(Figure 4C and supplementary Figure S2)* kept their initial rhythm. Hourly food intake in each group was modelled with the nlme R function; details of the model parameters are in *Supplementary Table 1*. Each of the three models yielded very significant R^2 (control, $R^2 = 0.59$; constant darkness, $R^2 = 0.58$; constant light, $R^2 = 0.36$; $p \approx 0$) and fitted the data well (dashed red line in *Figure 4*). This confirms the models were adequately describing

the 8400 measures (35 cohorts for 10 days of 24 hours). The light group displayed a damping coefficient γ significantly different from 0 (γ = -0.0068 ± 0.0004, $p < 0.001$) but not the control group (γ = -0.0001 ± 0.0002, p = 0.623). The group held in constant darkness did have a modest but significant damping, but it was much less important and not evident in *Figure 4C* (γ = -0.0006 ± 0.0002, p = 0.009), suggesting the absence of light only has a limited effect on food intake rhythms, whereas these are strongly affected by constant light. Consistently, the phases ϕ (peak consumption time) in control and constant darkness groups were very close (14.92 ± 0.13h and 14.78 ± 0.12h, respectively), as were their periods τ (24.17 ± 0.03h and 24.26 ± 0.02h, respectively). By contrast, as expected for the group held in constant light, both phase (15.80 ± 0.17h) and period (25.62 ± 0.07) were modified. Finally, consistent with result in *Figure 3B*, consummation peak (*A* in the model) was higher than control in the group held in constant light (3.07 ± 0.09 versus 1.63 ± 0.14), whereas in the constant darkness group it was slightly smaller (1.36 ± 0.07).

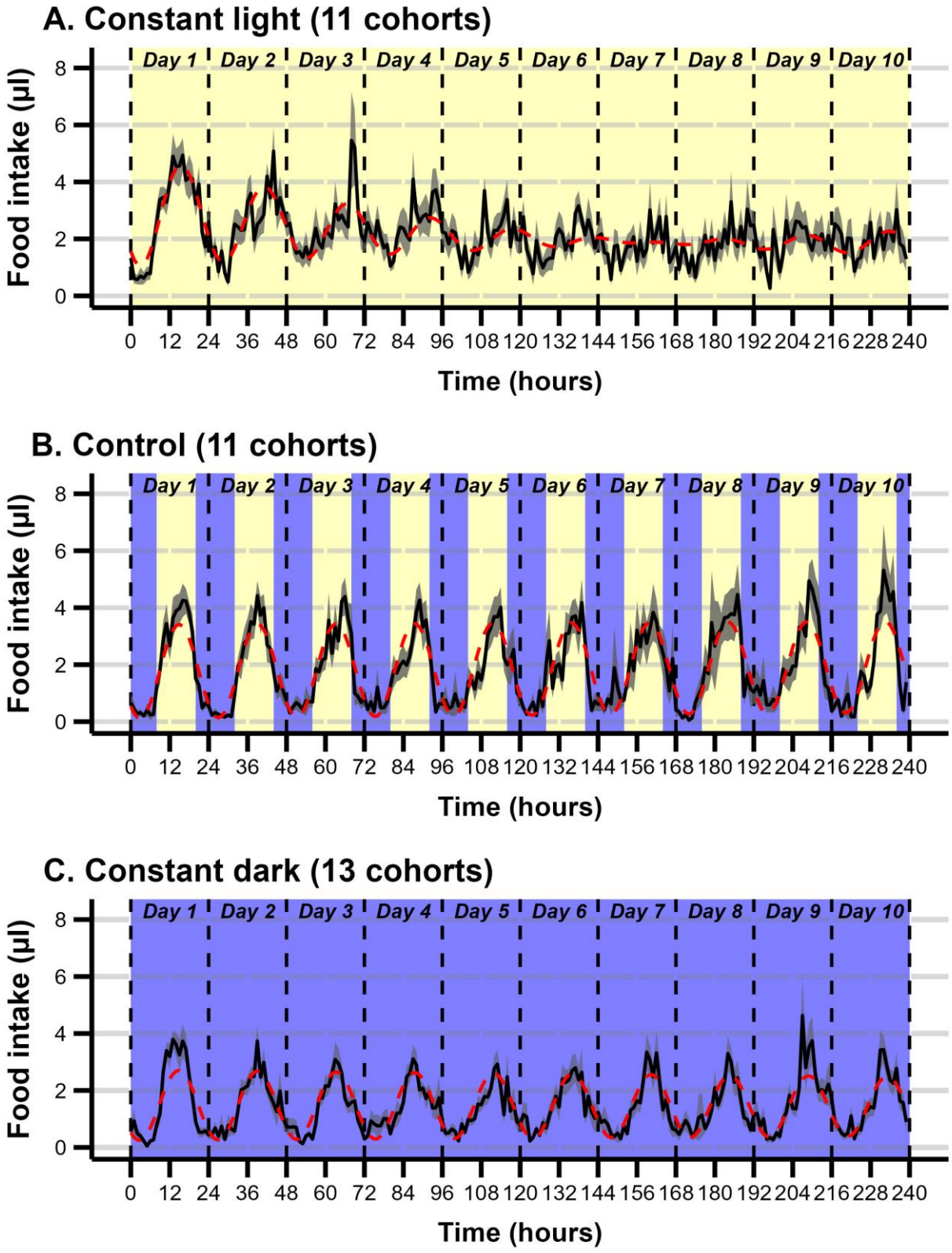

Figure 4. Hourly food intake averaged per living bee in the three conditions: constant light (A), control group (B) and constant dark (C). Data are the same as in *Figure 4B*, but not cumulated. Area in yellow or blue indicates periods of light and darkness, respectively. The shaded area around each curve is standard error of mean. Dashed red line is the fitted model. The time reported is the time elapsed since the onset of the experiment

4. DISCUSSION

Circadian rhythms of food intake in the BeeBox

In our experiments, we observed that the overall food consumption and mortality were linked to the photic environment: both were higher during the constant light condition and lower in constant darkness condition. Because we used identical rooms, experimental conditions running in parallel, and the same bee selection process, our results cannot be explained by particular bee characteristics or differential conditions; the effect of day duration at the time of each experiment was taken into account in analyses, allowing to dissociate them from the treatments. In our experiment, we measured a daily consumption between 35 and 50 µl per day per bee depending on the experimental condition. This level is comparable to the consumption observed with free available syrup dispensed with a feeder [98,99] and measured with the daily weighing of the feeder. This fact suggests that our feeding protocol (dispensing 2µl of syrup for each single flower visit) is comparable to a free feeding condition, i.e. feeding at a little of no cost [74].

Rhythmic consumption with a period longer than 24h has been observed and is largely maintained in a free run darkness test without environmental cues. To our knowledge, it is the first direct measure of circadian rhythms on food consumption in bees. Previous work has already used bees visiting feeders [18,45,49,55,56]; however, these studies did not measure food consumption but foraging intensity with locomotion or choice tests. As syrup gathered during foraging is not consumed directly but discharged and stored in the colony, syrup foraging do not reflect individual nutrition. Moreover, these studies limited the daily access to food to a short period of time. As in our protocol food was continuously available, we can exclude interpreting our results in terms of learning of time-dependent availability or as temporal control of behavior.

The level of consumption in the constant darkness condition demonstrates that even in dark, bees did not have problem to find the food source in the BeeBox. As a result, their failure to find the flower due to darkness is unlikely to explain differences between conditions. This conclusion is not surprising if we consider the relative darkness that is the rule for young bees in natural hives. Animals in constant darkness ate less and survived at least as much as control; they were probably less stressed. Limited food intake might affect bee longevity; however, while the positive impact of caloric restriction has been demonstrated in a lot of species [100], such link is still discussed in drosophila [101,102,102] and bees [103–105], for which sometimes opposing conclusions have been reached.

By contrast, in groups held in constant light the increased food consumption and mortality seem to be linked to increased locomotor activity [43–46]. Increased mortality has already been observed in constant light in drosophila [106,107]. It is possible that mortality could result directly from phototoxic damage: the insects could die from the direct action of light [108], with no relation

to rhythms or locomotion. This kind of phenomenon could contribute to the longer life expectancy of winter honey bees, which spend several months in the hive. This is also consistent with our observation that having preliminary experience with longer day durations also decreases survival (but not in the constant light group, were light exposure is saturating anyway); as we worked with young bees (which do not leave the hive), this experience might be indirect (i.e. through interactions with older, forager bees). The breakdown of the circadian rhythm could also be involved in increased mortality, although this is not the case in other insects [106]. Increased death could also occur because bees are more active possibly inducing a stress caused by lack of rest [109] or possibly because increased eating induces increased oxidative stress. However, our data does not permit to select one hypothesis and would require additional physiological investigation, which could be performed using the BeeBox.

We used young bees in our experiments; even in constant darkness young nurse bees display circadian rhythms in their locomotor activity, but they do not when they care for the brood in the presence of a light/dark cycle [110]. This indicates light has complex effects on rhythmic behaviours; what we observed with food intake is in line with their locomotor activity rather than with their brood caring, although the model did detect a modest damping. Contrary to drosophila's, hymenoptera's genome does not include the *Timeless* gene [111]. The timeless protein is destroyed by light, resulting in clock entraining. By contrast, the absence of this protein in bees prevents light to directly reset the clock; in general, in young bees, there is little oscillation of clock genes, even in regular light/dark cycle [110]. This would explain why a rhythm in food intake can exist whether or not light is present (i.e. in control and constant darkness groups). Excessive light would still alter the rhythm, probably due to a phototoxic stress as discussed above. This results in a damping of the rhythm as well as a drift of the period and the phase, which our models found to be similar across the treatments. The observed damping contrasts with Fuchikawa & Shimizu [112] who did not observed damping in the same light condition. However, contrary to our study where bees where exposed to the constant light condition during ten days, Fuchikawa & Shimizu exposed bees to light during only five days after five days of dark-light alternation. It is difficult to determine whether the damping observed in our experiment would have disappeared if the experiment had been preceded by a dark-light alternation condition, or if a prolonged exposure to light would have damped Fuchikawa & Shimizu's cycles. As we used *Apis mellifera* and Fuchikawa & Shimizu worked with *Apis cerana,* it is also possible that different bee species show different rhythms patterns.

The overall damping effect could result either from a general damping observed in most bees or to a loss of synchronization between bees with a variable shift of individual phase. As social contact is well known to activate rhythm synchronization between bees [43,50,113], the latter hypothesis is unlikely. Because all the experimental conditions were carried at the same time, and all bees from the

same colony were randomly affected to these conditions, we cannot explain our results with bee characteristics or environmental variations. Bees are known to exchange food through trophallaxis [114], so that caged bees do not show a uniform food consumption [115]. Preliminary observations we did before the experiment using marked bees also showed that some bees were highly active in the flower while others were waiting food transfers. As trophallactic exchanges are common in natural colonies, food exchanges in our cages can be seen as reflecting a natural phenomenon observed in bees living in groups [116,117]. Thus, our observation of circadian rhythm on food consumption only applies at the group level. The free run test performed in the dark suggests some kind of rhythm synchronization among active bees. This kind of synchronization is not surprising in social animals and has been previously observed in numerous species including bees [50,118–121]. Yet, investigating feeding rhythms at the individual level would still be interesting; this kind of protocol would require an improved version of the BeeBox.

Here, we studied how photic-regulated circadian rhythms modulate food intake, but food itself could be a rhythm regulator. The BeeBox could also be used to study food anticipatory activities with food available only during a short period of time each day, which gives the opportunity to study the use of periodic food as a "zeitgeber". With limited period of food availability initiated at any point of the day-night cycle, timing could also be investigated, as well as fixed interval schedules of reinforcement (i.e. food is unavailable for some time after feeding) [122]. Finally, a two-flowers conditioning chamber would permit to investigate time-place learning, for example, with one flower active at one time in the day, and the other one at another time. All these protocols fit perfectly within the possibilities of the BeeBox, provided the adequate software is added to the microcontroller board.

Advantages, limitations, and potential uses of the BeeBox

In addition to making it possible to show circadian feeding patterns, the BeeBox includes several features that greatly improve the standard feeding protocol that uses caged bees with free access to artificial nectar. First, the particular shape of the sliding cover makes it possible to introduce bees without anaesthesia (*Figure 1C*). As it is known that the anaesthesia done with cold or carbon dioxide may affect honey bees [123,124], removing anaesthesia from the protocol is an interesting improvement. Second, with our device, mortality is assessed hourly, even during darkness, with automatic pictures that do not need the presence of an observer. When mortality is low, sampling rate is not critical, but with high and fast mortality following a pesticide treatment, for example, a finer resolution for mortality assessment would be useful [125]. Our device offers this possibility, and assessment time shorter than 1 hour would be possible too; we can even hypothesize a circadian mortality in bee colonies [126]. Third, contrary to the standard bee cage device, our protocol is 100% automatized and there is no need to involve the experimenter (except when refilling food reserve).

Apart during the introduction of the bees in the chamber, there is also no interaction between the experimenter and the bees. Developing automated tools that minimize the role of the experimenter is an important step toward the standardization of protocols [127] and the reproducibility of results [128]. Finally, with the same device and protocol, it is possible to simultaneously measure mortality, food consumption, and circadian rhythms in integrated experiments [44,66].

The BeeBox provides many advantages but has some limitations too. Contrary to other studies [129,130], our IR sensor detects bee entering flowers rather than proboscis extension [75]. This choice has been made to request a substantial energy expense from the bee for each response. Yet, it would be possible for some bees to activate syrup dispensing without consuming it (by entering the flower without drinking) so that the measure of consumption based on dispensed volumes would give overestimated values. To avoid this problem, we dispensed only 2µl drops of syrup. This volume has been empirically defined in such a way that it prevented syrup accumulation in the flower during successive visits.

Operant conditioning is a well-known form of learning [131,132] during which the occurrence of a behaviour is modulated by its outcome [132,133]. In the BeeBox, the trained response is flower or tube entrance while syrup acts as a reinforcer of this response. But as the syrup is given at the bottom of the tube, the reinforced response somewhat overlaps the syrup consumption response. Moreover, the stimuli associated with the operant response and with the reward are spatially combined. In pigeon, this protocol would be similar to reinforce with grain the introduction of the head inside the feeder instead of pecking a key [134]; this is also similar to nose poking conditioning in rodents [135]. Consequently, our protocol could perhaps result in a mix of sign tracking and goal tracking [136], the latter being Pavlovian conditioning rather than operant conditioning. As cues and rewards are also simultaneously presented in natural flowers, with which natural selection has operated, the question of how the two types of conditioning interact in natural or artificial flowers could also be explored with the BeeBox in a cognitive ecology investigation. Many protocols could also be envisioned to manipulate the syrup properties (sucrose concentration or composition) in simple flower or choice tests [68] to study the feeding strategies or nutrient choice [137,138]. Thus, the BeeBox will provide a very useful experimental set-up to perform this type of laboratory study, and also probably many others.

In addition to studies having the goal to understand the basic determinants of bee behaviour [139], our BeeBox platform could also be used in numerous applications related to several scientific fields. For example, our conditioning chambers could offer an improved alternative to the standard bee cage protocol [66,98,140] in the assessment of pesticide toxicity. Our results question the choice of conducting such tests only in the dark conditions, as this generates the lowest level of mortality and food consumption [140]. This fact may be also problematic in chronic toxicity assessment

because in darkness, the bees would consume the smallest dose of the tested pesticide at the end of the test. All this suggests that the LD50 (median lethal dose) and/or the LC50 (median lethal concentration) may be poorly measured in a number of conditions based on an arbitrary protocol choice, leading to inappropriate conclusions about the pesticide toxicity. The repeated and chronic consumption of pesticides could be investigated by adding a known concentration of pesticide in the syrup tank and running the experiment during a couple of weeks [98]. It would also be easy to define a nutritional stress [98], either by limiting reinforcer amplitude, or by increasing the amount of flower entrance required to get food (fixed-ratio reinforcement schedule); this would simulate nectar depletion of the environment.

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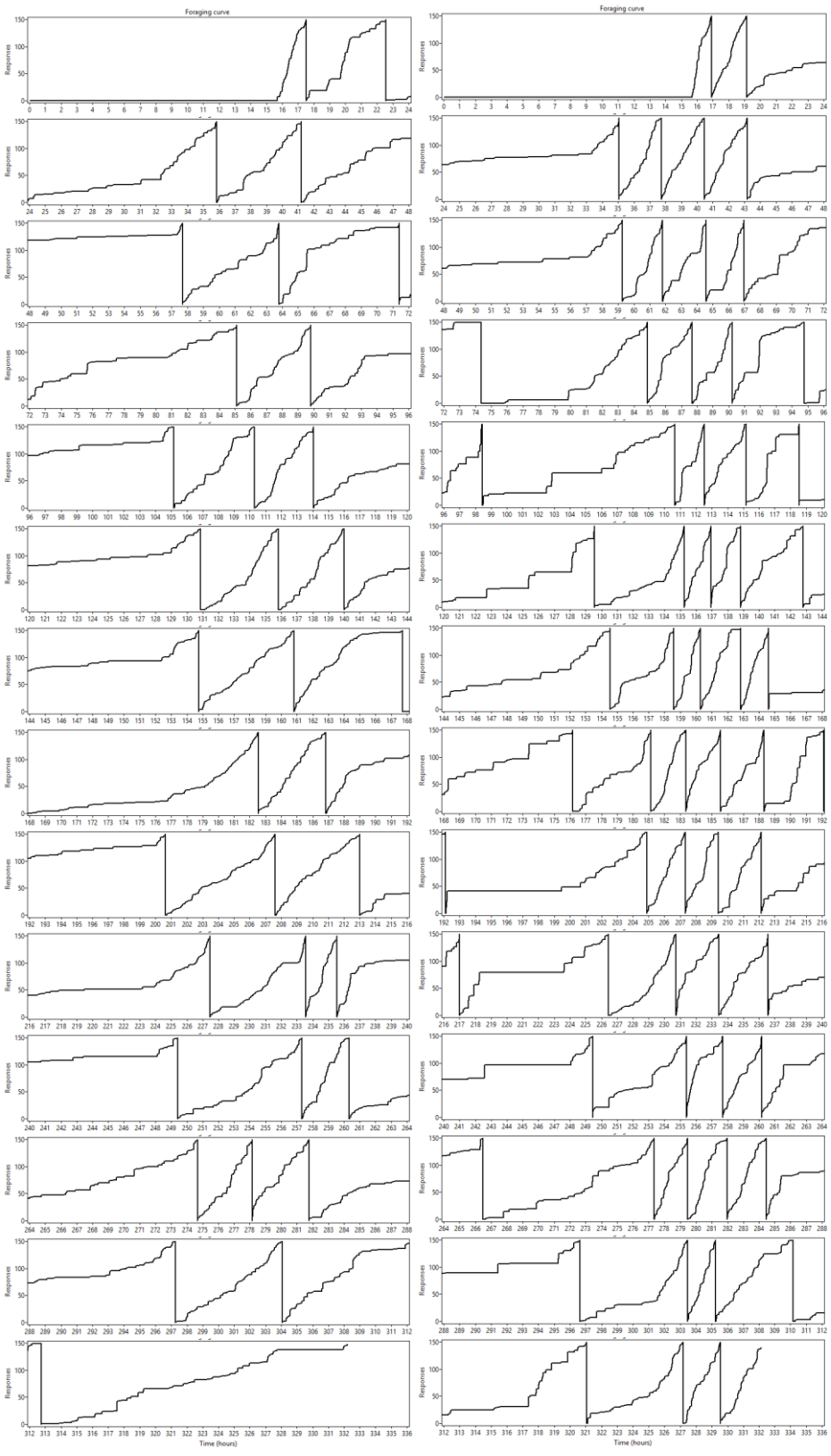
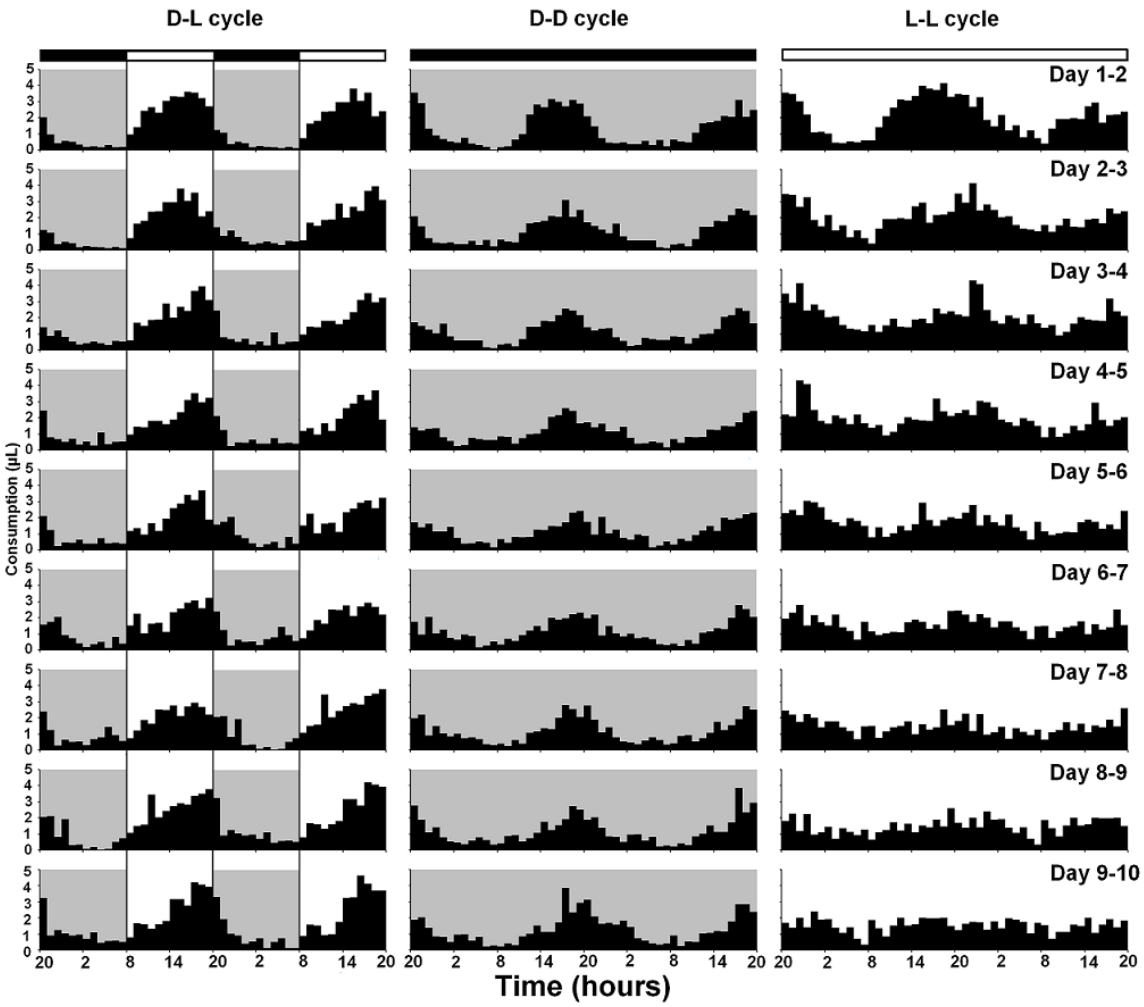
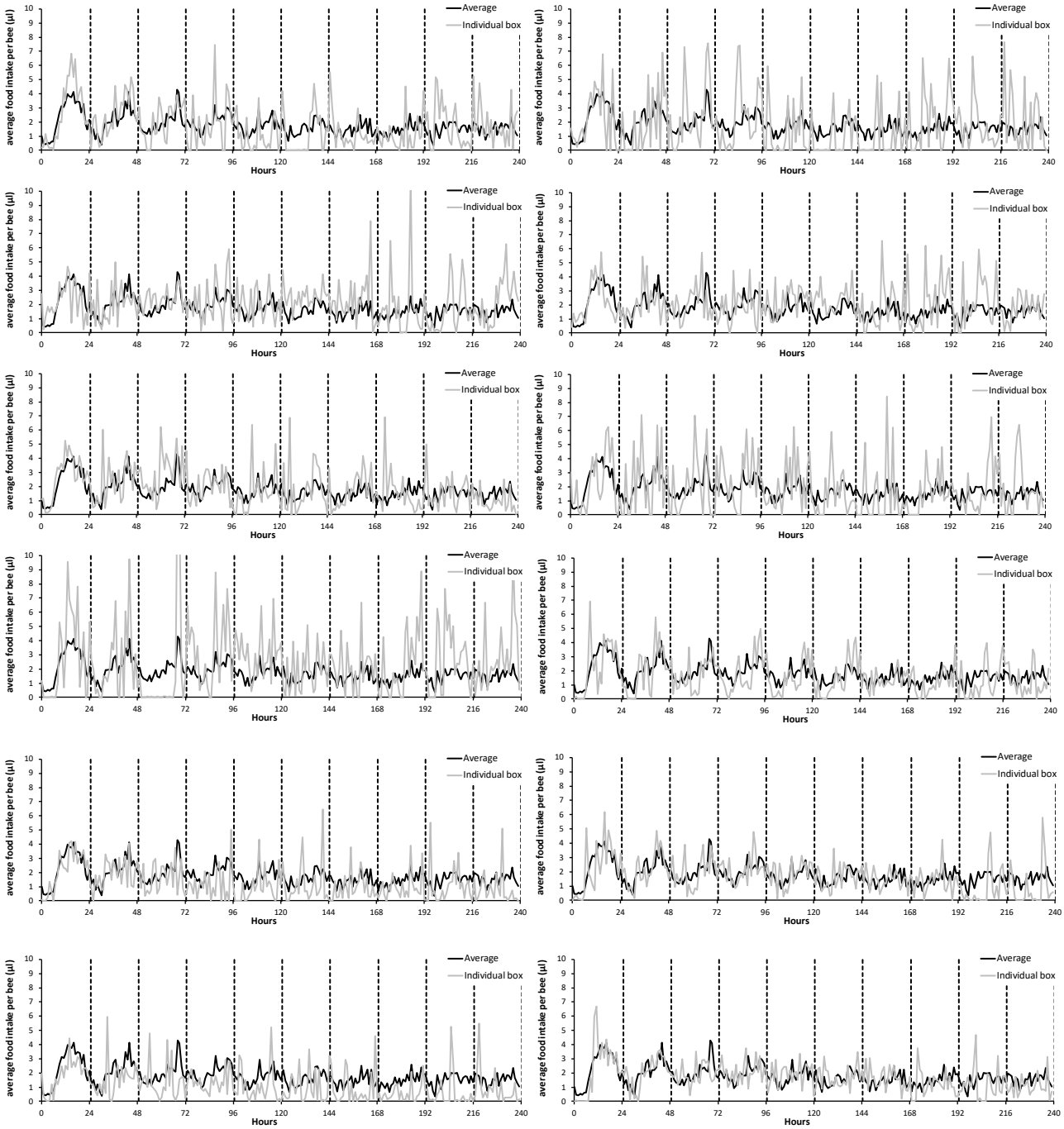


Supplementary Figure S1. Two samples (left and right) of cumulative curves for two groups of bees. Each level corresponds to 24h of experiment. The vertical axis shows cumulative responses with a reset each time the response number exceeds the vertical amplitude axis. These curves are directly drawn by the data analysis software during the experiment.



Supplementary Figure S2. Average actogram for all the conditions and bees of the experiment. The time reported is the time elapsed since the onset of the experiment. Gray areas show darkness intervals. Similar to figure 3, we can see rhythmic consumption with a maximum during the light period for the control (D-L cycle) and the always dark (D-D cycle) conditions. Damping of oscillations is detectable in the always light condition (L-L cycle).



Supplementary Figure S3. Single cohort data in the L-L condition. For comparison, the average data is also shown on each graphic. The vertical lines are separations between successive days.

Supplementary Table 1

	Estimated values	p-value
γ (damping) for control	-0.0001 ± 0.0002	0.623
γ for constant dark	-0.0006 ± 0.0002	0.009
γ for constant light	-0.0068 ± 0.0004	< 0.001
A (peak amplitude, in µl) for control	1.63 ± 0.14	< 0.001
A for constant dark	1.36 ± 0.07	< 0.001
A for constant light	3.07 ± 0.09	< 0.001
ϕ (phase, in hour) for control	14.92 ± 0.13	< 0.001
ϕ for constant dark	14.78 ± 0.12	< 0.001
ϕ for constant light	15.80 ± 0.17	< 0.001
τ (period, in hour) for control	24.17 ± 0.03	< 0.001
τ for constant dark	24.26 ± 0.02	< 0.001
τ for constant light	25.62 ± 0.07	< 0.001

The p-value test the null hypothesis that the coefficient is equal to zero. Values are provided with their standard error.